DISS. ETH NO. 21284

MISMATCH REPAIR DEPENDENT PROCESSING OF O6-METHYLGUANINE ADDUCTS IN *XENOPUS* EGG EXTRACTS

A dissertation submitted to
ETH ZURICH

for the degree of
Doctor of Sciences

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2013

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1 Zusammenfassung

Die Fehlpaarungsreparatur (auf englisch, mismatch repair oder MMR) einer Zelle hat die Aufgabe, Mutationen, die durch Fehlbasenpaarung in unserem Genom entstehen, zu verhindern. Demzufolge korrigiert es biosynthetische Fehler in unserer DNS (Fehlpaarung von Basen, Insertionen und Deletionen) die waehrend der Replikation eingebaut werden koennen. Ihre Aufgaben umfassen nicht nur die Überprüfung der Lesefunktion der Polymerasen waehrend der Replikation, sondern auch die meiotische und mitotische Rekombination, die Stabilität von repetitiven Trinukleotiden und die Reaktion auf DNS-Schäden.

S_N1-methylierenden Substanzen wie Temozolomid und Dacarbazin und aehnlichen nicht-klinischen Substanzen wie N-Methyl-N-nitro-Nitronitrosoguanidin (MNNG) und N-Methyl-N-Nitrosoharnstoff (MNU) verursachen eine Vielzahl von Addukten. Obwohl die meisten davon veraenderte Stickstoffatome von Purin-basen sind, ist die Toxizitaet dieser Substanzen nicht darauf zurückzufuehren, sondern auf O6-methylguanin (^{me}G). Dieses Addukt ist jedoch fuer weniger als 8% der Methylierungen in der Zelle verantwortlich und ist trotz dessen das einzige toxische Addukt.

Die Fehlpaarungsreparatur spielt eine wichtige Rolle bei den zellulaeren Antworten auf diese Wirkstoffe. Inwiefern die Prozessierung der ^{me}G-Addukte bei MMR waehrend der Replikation für den Zelltod im zweiten Zellzyklus verantwortlich ist, bleibt jedoch eine offene Frage.

Es gibt zwei Hypothesen, die versuchen Klarheit über das Thema zu bringen. Die erste besagt, dass die repetitiven Reparatur-versuche der Fehlpaarungsreparatur den Schaden zu korrigieren, der sich aber im Eltern-doppelstrang befindet und deshalb nicht korrigierbar ist, den Zelltod bringen. Dies wurde die Tatsache erklaeren, dass zellulaere Checkpoints erst im zweiten Zellzyklus deutlich aktiviert werden, und nicht gleich nach der Fehlpaarungs-erkennung. Das zweite Model behauptet, MMR koenne direkt mit zellulaeren Checkpoint-Proteine interagieren und somit auf eine sehr direkte Weise den Checkpoint aktivieren. Dieses Model basiert auf wissenschaftlichen Publikationen, die Interaktionen zwischen MMR Proteinen und Checkpoint-Proteinen nachgewiesen haben. Eierextrakte aus dem Frosch *Xenopus laevis* sind eine etablierte Methode, um Replikationsabhängige Signalkaskaden zu untersuchen. Sie erlauben die *in vitro*

Replizierung von spezifische DNS Vorlagen und dienen dazu, die Dynamik waehrend der Replikation unter verschiedenen Bedingungen naeher zu untersuchen. In unserem Fall war die Replikation nicht-methylierter und methylierter DNS von Interesse. In dieser Studie wurde wissenschaftlich nachgewiesen, dass MMR Proteine und Checkpoint-Proteine nicht unbedingt gleichzeitig zu ^{me}G in der DNS rekrutiert werden. Darueber hinaus wurde festegestellt, dass keine MMR-abhänginge Aktivierung eines Checkpoints sofort nach Erkennung der ^{me}G-Substrate stattfindet. Dabei zeigte sich, dass ein transienter Checkpoint nach Erkennung von MMR Substraten zwar zu sehen ist, aber dies nicht ^{me}G spezifisch ist. Hinzu kommt, dass MMR-abhängige Zwischenprodukte in der DNS nachgewiesen werden konnten. Diese entstehen durch die Reparaturversuchen des MMR Systems von ^{me}Gs in der DNS und wirken sich tatsächlich negativ auf eine weitere Replikation aus. Zum Schluss sind wir zu der Erkenntnis gekommen, dass die Erkennung von ^{me}G-Substraten bei der Fehlpaarungsreparatur nicht ausreichend ist, um das Phänomen des Zelltodes im zweiten Zyklus zu erklären.

2 Abstract

The mismatch repair system is part of the cellular machinery assigned to obfuscate mutations that might perturb canonical base pairing in the genome. As such it corrects DNA biosynthetic errors (base-base mismatches and insertion-deletion loops) that arise during replication. Its functions extend beyond proofreading errors of replicative polymerases, to meiotic and mitotic recombination, triplet repeat stability and the DNA damage response.

S_N1-type methylating agents such as temozolomide and dacarbazine and their non-clinical counterparts N-methyl-N'-nitro-N-nitrosoguanidine **(**MNNG) and N-methyl-N-nitrosourea (MNU) generate a variety of lesions. Of these, more than 80% are modified purine nitrogen atoms, but their toxicity appears to result from the processing of O6-methylguanine (^{me}G) despite the fact that this lesion only accounts for up to 8% of the generated lesions.

MMR has been found to be a major player in checkpoint and apoptotic responses attributed to this type of damage. To what extent MMR-dependent processing of the adducts during replication is the ultimate cause of cell arrest/death remains a topic of discussion. Currently, two models tackle the involvement of MMR in the processing of methylation-induced damage. The first posits that MMR activates damage signaling indirectly through its repetitive attempts at processing ^{me}G lesions in the template strand during replication. This would accommodate the fact that the MMR-dependent cell cycle arrest in G2/M is only observed in the second cycle and not immediately after ^{me}G recognition by the repair machinery. Alternatively, binding of the MMR system to the mismatch is sufficient to trigger kinase activation. This model is mainly substantiated by reported interactions between MMR and the ATR checkpoint machinery upon treatment with S_N1-type alkylators.

The *Xenopus* egg extract technique has surfaced as a unique tool for analysis of replication-dependent DNA damage signaling. This experimental system initiates replication on defined templates within a biochemical setup and is able to closely follow the dynamics of template replication. In our case, it enabled the study of MMR activity during replication of alkylation damaged and undamaged DNA templates.

Here, we show that recruitment of the ATR checkpoint machinery to sites of alkylation damage, or more specifically O6-methylguanine, is not simultaneous with that of MMR proteins. Furthermore, an accumulation of ^{me}G/T mispairs during replication, a preferred MMR substrate, is in itself not enough to trigger prolonged checkpoint activation. Finally, we show that gaps arise in DNA due to MMR-dependent ^{me}G processing behind the fork and that they largely escape checkpoint surveillance. In conclusion, these results substantiate that mere presence of either ^{me}G/C or ^{me}G/T in the DNA during a first round of replication is an unlikely cause for checkpoint activation and the G2 arrest seen during the second cell cycle, and that it is rather the presence of ^{me}G-dependent gaps which can be deleterious to further replication.

3 Introduction

"Just as physics and chemistry are based on molecules and atoms, even so the biological sciences must penetrate to these units in order to explain by their combinations the phenomena of the living world" Hugo de Vries (taken from "The Century of the gene", Evelyn Fox Keller, Harvard University Press, 2002).

3.1 Eukaryotic DNA replication

The sequencing of the human genome, completed in April of 2003, marked a breakthrough in 21st century biology. Science has come a long way since Watson, Crick, Wilkins and Franklin first identified DNA as a base-paired double-helical structure in the 1950s. How this information, our genome, is passed on from cell to cell, generation to generation, producing identical copies of the original double-stranded DNA is studied in the field of DNA replication. Replication is the process by which a cell copies its DNA during a synthesis phase (S phase) prior to mitotic cell division (M phase). It is semi-conservative in the sense that it produces two copies that each contain one of the original strands and one new strand. Pertinent to this thesis is a focus on eukaryotic DNA replication and the replication machinery made up of a plethora of evolutionarily conserved proteins. Although, as in any field of study, there are still open questions pertaining to all the steps involved, significant progress has been made in its understanding and I will summarize the most crucial aspects and novel discoveries of the past years.

DNA replication requires pre-replication complex (pre-RC) formation just prior to S phase and origin firing at the start of it (see Fig. 1). The assembly of the pre-RC starts with the origin recognition complex (ORC) complex (ORC1, 2, 3, 4, 5 and 6) recognizing DNA origins and the loading of the replicative helicase in late M of the cell cycle. This hexamer composed of the minichromosome maintenance (MCM2-7) proteins loads as a double hexamer [1, 2] in a manner dependent on ORC, cell division cycle 6 (Cdc6) and Cdc10 dependent transcript 1 (Cdt1) [3]. Assembly of the pre-RC on origins occurs only during late mitosis to early Gap 1 phase (G1) in order to prevent rereplication. Activation of the pre-RC and origin firing is not detected until S phase and involves expression and activity of two kinases, cell division cycle 7 (Cdc7) and cyclin dependent kinase 2 (Cdk2), as well as recruitment of proteins such as DNA topoisomerase 2-binding protein 1 (TopBP1),

Cdc45, MCM10 and the GINS (from the Japanese go-ichi-ni-san meaning 5-1-2-3, after its four related subunits Sld5, Psf1, Psf2 and Psf3) complex.

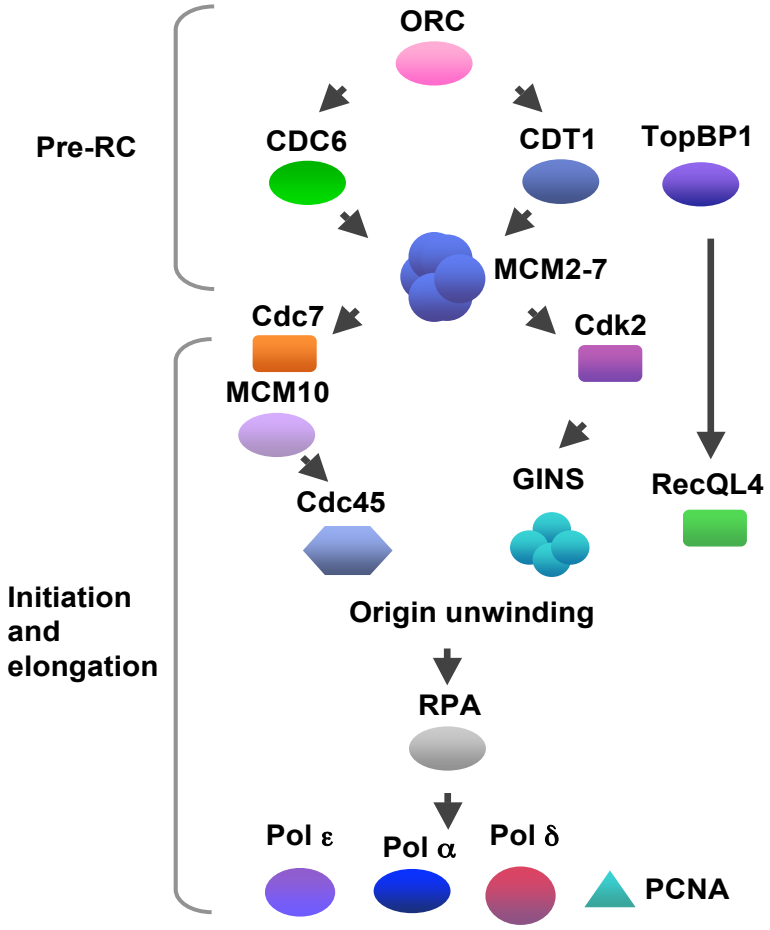


Figure 1. Origin unwinding and elongation at eukaryotic replication origins. Formation of the pre-RC complex preceeds origin firing. This involves binding of MCM2-7 proteins in a manner dependent on ORC, Cdt1 and Cdc6 in late M/G1. Activation of DNA replication by the Cdk2 and Cdc7 kinases triggers binding of additional factors such as Cdc45, MCM10, GINS, RPA and the replicative polymerases (α, ε, δ). Proteins involved in elongation of the DNA include RFC, PCNA and MCM10. Adapted from [4]

Recently identified TopBP1 interactors, GEMC1 (geminin coiled-coil domain-containing protein 1) and Treslin have a role in loading of Cdc45 onto the chromatin [5-7]. With regards to the kinase activity neccessary for initiation, Cdc7 phosphorylates the MCM2-7 subunits, which is thought to facilitate loading of factors such as Cdc45 and MCM10. Cdk2 targets have not been clearly identified although the ReQL4 protein, a homolog of

the yeast Cdk2 target Sld2/Drc1 that is required for loading of the RPA heterotrimer after origin unwinding, might be a target [8, 9].

Formation of an active replication fork is achieved through the recruitment of the replicative polymerases α, ε, δ. Pol α primase primes DNA synthesis on both strands, whereas Pol ε and pol δ elongate the leading and lagging strand, respectively [10]. TopBP1 and GINS might cooperate in loading pol ε onto origins, whereas Ctf4/And1 and MCM10 were shown to be important for pol α stability and replicative function. In 2011, a study recapitulating *in vitro* replication in yeast observed that MCM10 depletion affects leading but not lagging strand polymerase recruitment. This could be observed by monitoring pol δ and pol ε association with chromatin [11].

Although a corelation between replication and histone acetylation had been noted previously [12] it is now known that the post-translational modification me2H4K20 (dimethylated lysine 20 on histone 4) is bound by the BAH (bromo adjacent homology) domain in ORC1, directly linking the methylome to DNA replication. me2H4K20 is enriched at replication origins and possibly aids selection of replication origins [13]. Also of note, although ORC binding sites have been proposed in different eukaryotic organisms, there is no consensus as to the nature of the DNA sequence and the way in which ORC is recruited to DNA. With regards to this, an ORC interacting protein was identified in a mass spectrometric analysis of ORC-interacting proteins. ORC-associated/leucine rich repeats and WD repeat domain containing 1 (ORCA/LWD1) is crucial for ORC association to chromatin and has been shown to be capable of artificially recruiting ORC to a specific genomic locus [14]. Other players involved in DNA replication have been reported. They can be proteins, for example, MCM8 and MCM9, required for replication elongation but not initiation in *Xenopus* egg extracts [15, 16] or perhaps more surprisingly, RNA.

Small, stem-loop RNAs, Y RNAs, first identified as part of Ro ribonucleoprotein particles in higher eukaryotes, interact with ORC and other pre-RC components and seem to be accordingly required for replication initiation [17]. Intriguingly, many of these replication proteins are known to function outside of replication during S phase. For example, MCM8 and 9 were shown to also play a role in repair of lesions requiring homologous recombination [18], highlighting the crosstalk between the replication machinery and DNA repair pathways.

3.2 Cellular DNA repair pathways

DNA damage accumulates in a cell through exposure to several types of exogenous and endogenous agents such as sunlight, cigarette smoke, reactive oxygen and nitrogen species, etc. In response to this asault, cells have developed vital tolerance and repair mechanisms to ensure the perpetuity of their DNA. The following chapter of this thesis will focus on the description of the main DNA repair pathways of a cell.

3.2.1 Direct DNA damage reversal

This pathway refers to repair systems in a cell which lack the broad substrate specificity and the requirement for multiple steps to accomplish repair. It is simple and often narrow in its substrate choice. The primary type of lesions addressed by direct DNA damage reversal are generated by alkylating agents. Endogenously, the metabolite S-adenosylmethionine (SAM), is known to weakly methylate DNA. Significant alkylation damage in DNA is generated upon exposure to agents such as the S_N1-type alkylators N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), N-methyl-N-nitrosourea (MNU) or the S_N2-type alkylator methyl methanesulfonate (MMS). S_N1-type alkylators cause a variety of N- and O-alkylated products. MNU and MNNG, for example, generate about 70% N7-methylguanine, 9% N3-methyladenine, 8% O6-methylguanine and 2% N3-methylguanine, amongst others [19]. While most of these adducts are efficiently repaired by the multistep base excision repair (BER), O6-methylguanine (^{me}G) is a well known example of a lesion that can be repaired in a single step. In humans, it requires O6-methylguanine-methyltransferase (MGMT), also referred to as alkylguanine transferase (AGT). Both the human and the *E. coli* version of this protein transfer the methyl group from the guanine to an internal cysteine residue in what is known as a suicide reaction since it leads to the irrevocable degradation of the enzyme [20]. The *E. coli* protein, Ada, is composed of an N- and C-terminal part separated by a hinge region. The C-terminal part repairs ^{me}G and O4-methylthymine while the N-terminal part demethylates methyl phosphotriester lesions in DNA. This is part of an adaptive response to the increased sensoring of damage [21]. A second O6-methylguanine methyltransferase, Ogt, is constitutively expressed and handles lower amounts of the modified guanine even though it shows preference for O4-methylthymine [22]. In the clinic, inhibition of MGMT using pseudosubstrate nucleotide analogs such as O^6-(4-bromothenyl)guanine (Patrin-2) and O6-benzylguanine (BG) is of particular interest when attempting to sensitize cells to

killing by alkylating agents [23]. Another set of lesions generated by alkylators, in this case, S_N2-type alkylators, are N1-methyladenine and N3-methylcytosine [24]. These are repaired via oxidative demethylation by alkB alkylation repair homolog 2 (ABH2) and ABH3, human homologues of the *E. coli* AlkB protein. However, whereas ABH2 prefers double-stranded DNA, ABH3 and AlkB favour single-stranded DNA and RNA [25].

3.2.2 Mismatch repair

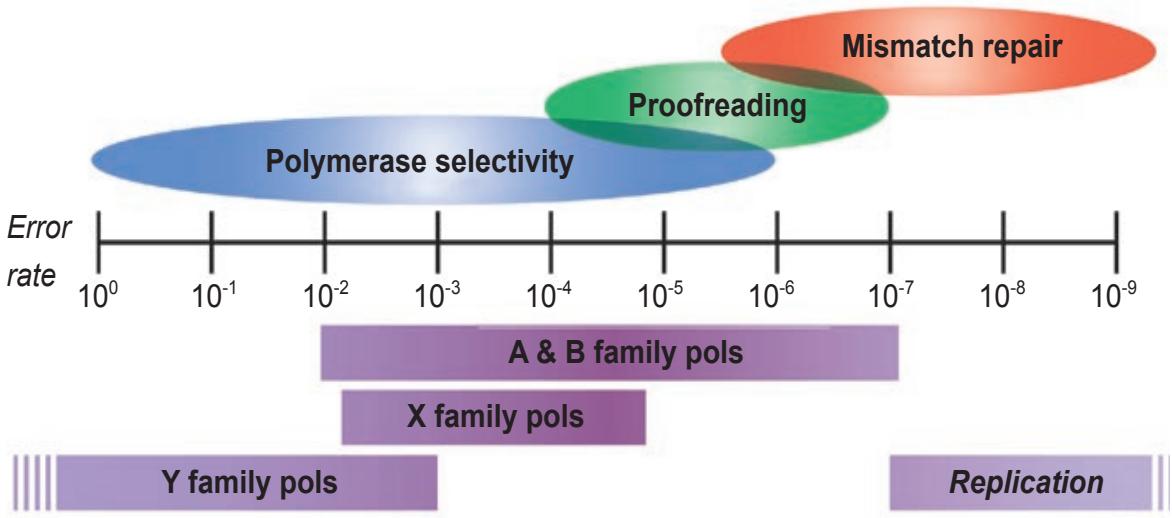


Figure 2. Determinants of replication fidelity. The relative contribution of the three main components of replication fidelity, the estimated error rate for different families of polymerases and the mutation rate of the *in vivo* complete replication process. Taken from [26].

The mismatch repair (MMR) framework is designed to improve the replication fidelity of replicative polymerases by several orders of magnitude (see Fig. 2), be it by correcting base-base mismatches or by counteracting polymerase slippage which leads to the formation of insertion/deletion loops in DNA [27]. The accuracy of eukaryotic DNA synthesis is estimated to be at least as efficient as that of *E. coli* or bacteriophage replication. That is, with a base substitution error rate ranging from 10^{-7} to 10^{-8} or, in other words, lower than one error for every billion or more base pairs [26, 28, 29].

A better understanding of mismatch repair is aided by a comparison between the prokaryotic and eukaryotic mismatch repair machineries, thereby identifying important similarities and adaptation from a less evolved to a more evolved organism. A brief summary summary of the two systems is offered below and Table 1 provides an

overview of proteins involved in MMR in *T. thermophilus, E. coli, S. cerevisiae and H. sapiens.*

Molecular function	*E. coli*	*S. cerevisae*	*H. sapiens*
Mismatch recognition	MutS	MutSα, MuSβ PCNA	MutSα, MuSβ PCNA
Strand incision	-	RFC MutLα, MutLγ *2	RFC MutLα, MutLγ *2
Strand incision	MutH	-	-
Match making	MutL	MutLα, MutLβ, MutLγ	MutLα, MutLβ, MutLγ
Strand excision (single strand DNA binding)	SSB	RPA	RPA
Strand excision (exonuclease)	RecJ, Exo1, ExoX, ExoVII	EXO1 *3	EXO1 *3
Strand excision (helicase)	UvrD	-	-
Repair synthesis	DNA pol III	DNA pol δ	DNA pol δ

Table 1. Distribution of MMR proteins. *1 Involvement has not been confirmed yet. *2 The endonuclease activity of MLH3 has not been confirmed biochemically. *3 In yeast and humans EXO1 has a 5' Flap-endonuclease activity in addition to 5'-3' exonuclease activity. Taken from [30].

In general, MMR machineries are equipped with the ability to distinguish mismatches in DNA, discriminate the parental and daughter strands during replication as well as the ability to act bidirectionally.

3.2.2.1 Prokaryotic mismatch repair

E. coli MMR has been studied extensively both biochemically and genetically. It has even been reconstituted *in vitro* using recombinant proteins [31]. This system requires the following components: MutS, MutL, MutH, DNA helicase II (UvrD), four exonucleases (ExoI, ExoVII, ExoX and RecJ), single-stranded DNA binding protein (Ssb), DNA

polymerase III holoenzyme and DNA ligase [32]. Mismatches are recognized by the MutS homodimer and an interaction with the MutL homodimer [33] is neccessary for enhanced activation of the MutH restriction endonuclease. MutH utilizes transient hemimethylated dGATC sites to introduce an entry point for excision of the error-containing daughter strand [34, 35]. This sequence is normally methylated at the N6 position of adenine in *E. coli* DNA. Depending on the position of the break relative to the mismatch, the aforementioned 5'-3' (ExoVII or RecJ) or 3'-5' (Exo1 or ExoX) exonucleases [36] and the UvrD helicase [37] remove this strand, and a new strand is synthesized and sealed by DNA polymerase III and DNA ligase.

3.2.2.2 Eukaryotic mismatch repair

Several human MMR proteins have been identified based on their homology to the *E. coli* proteins. In this system, however, the main players act as heterodimers and have diversified into more substrate-specific proteins. MutS homologue 2 (MSH2) heterodimerizes with MSH6 or MSH3 to form MutSα or MutSβ, respectively. Likewise, MutL homologue 1 (MLH1) heterodimerizes with post-meiotic segregation 2 (PMS2) (MutLα), PMS1 (MutLβ) and MLH3 (MutLγ). Of the three, only MutLα has a clear function during replication. MutLβ is involved in meiotic recombination and MutLγ function remains unsolved [38-41]. The minimal human MMR system was reconstituted several years ago using recombinant proteins. It is composed of MutSα or MutSβ, MutLα, Exonuclease 1 (EXO1), replication factor C (RFC), proliferating cell nuclear antigen (PCNA), replicating protein A (RPA), polymerase δ and DNA ligase I [42-44].

As in *E. coli*, MMR starts with recognition of a mispaired base in the daughter strand during replication (see Fig. 3). How this activity is targeted is not fully clear but, it is generally believed that strand breaks in the leading and lagging strand due to damage correction by other DNA repair pathways or the presence of 3' and 5' termini at Okazaki fragments in the lagging strand, could act as discrimination signals [45]. MutSα recognizes base-base mismatches and short insertion/deletion loops. MutSβ binds larger IDLs of two to three extrahelical nucleotides [46]. MutLα interacts with the MutS heterodimers [47] and this complex coordinates 5'-3' excision by EXO1 [48, 49].

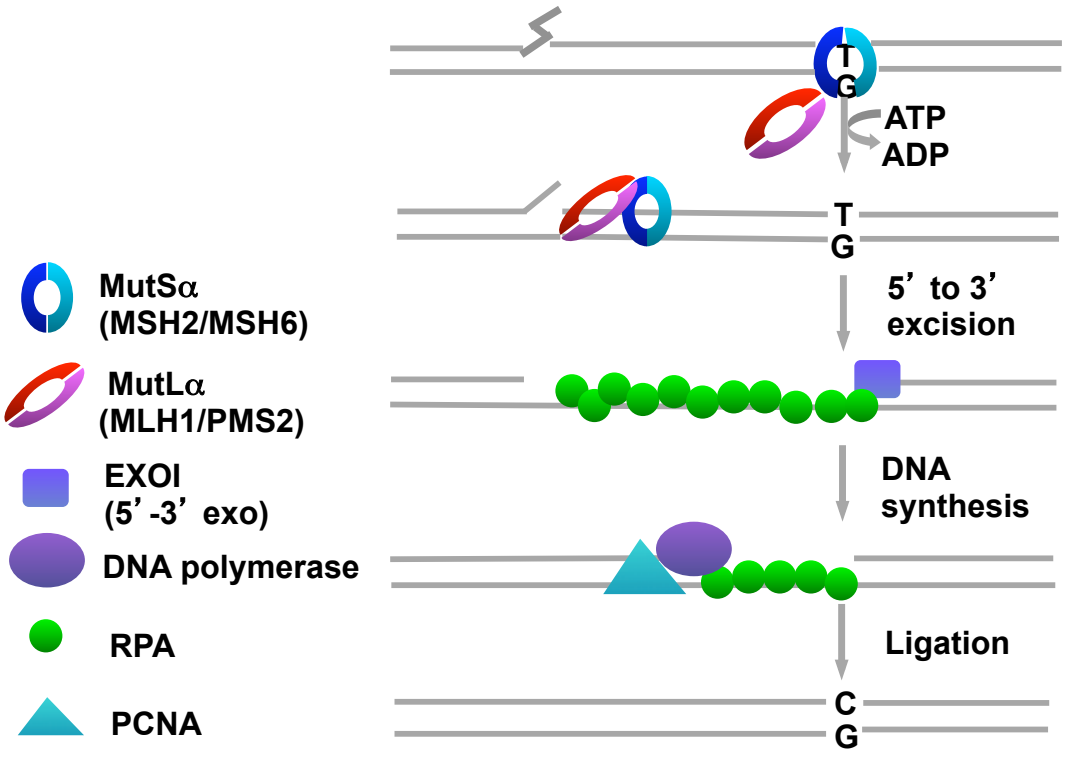


Fig. 3. Schematic representation of eukaryotic mismatch repair (MMR). MutSα (MSH2/MSH6) binds a mismatch, recruits MutLα (MLH1/PMS2 heterodimer) and translocates along the DNA until it encounters proliferating cell nuclear antigen (PCNA) bound at the 3' terminus of the nick. This ternary complex introduces additional breaks in the nicked strand, where exonuclease 1 (EXO1) is loaded. EXO1 generates a long single-stranded gap which is stabilized by RPA and then filled in by the PCNA/DNA polymerase complex. The remaining nick is then sealed. Adapted from [50].

Since 3'-5' exonuclease activity in this system has not been identified, the bidirectionality of eukaryotic mismatch remained a mystery until it was shown that the PMS2 subunit of MutLα and yeast MutLα (MLH1/PMS1) contain a PCNA-activated endonuclease activity in their C-termini that can introduce single-strand breaks in the vicinity of the mismatch [51-53]. RPA stabilizes the single-stranded DNA region generated by EXO1 excision, and resynthesis is taken over by polymerase δ and ligase I [54-57].

3.2.2.3 Mismatch recognition

MutS proteins have an ATPase domain that is reponsible for binding and hydrolysis of ATP [58]. This enzymatic activity is believed to be important in providing MutS with a conformational switch so it may translocate along the DNA attempting to find

discontinuities that will support excision of the mismatched nucleotide [59]. MSH2 has two distinct interaction regions for both MSH3 and MSH6 (see Fig. 4) at its N- and C-termini. ATP binding domains are found at its C-terminus of all three proteins. In contrast, MLH1 interacts with PMS1, PMS2 and MLH3 only through its C-terminus and has N-terminal ATP-binding sites. MSH2, MSH3 and MLH1 have been found to interact with EXO1 and this interaction is important for the excision step during MMR [60, 61]. A further interaction that is important for MMR, at least *in vitro*, is that between both MutSα or MutSβ and PCNA. PCNA is important in mismatch repair as an elongation factor for polymerase δ and also in earlier steps of the repair [62, 63]. A conserved motif QXX(L/I)XXFF at the N-terminus of MSH6 and MSH3 has been found to be crucial for this interaction [64-66].

Both bacterial MutS and human MutSα contact the mismatched base through a conserved phenylalanine residue (Phe36 in *E. coli* and Phe432 in human MSH6) that intercalates from the minor groove [67]. An adjacent glutamate residue (Glu38 in *E. coli* and Glu434 in human MSH6) forms a hydrogen bond with the mismatched base and is also crucial for mismatch recognition. In order for this close contact to be made available to the mismatch repair machinery, crystallographic and biochemical data suggest a certain a priori flexibility of the DNA at places that contain mismatched nucleotides [67, 68]. Indeed, mispaired bases weaken base stacking interactions and this, in turn, could lead to a propensity toward DNA bending [69]. Paradoxically, although the G:T mismatch causes the least amount of double-helix distortion, it remains one of the most efficiently repaired MMR substrate [70, 71].

Crystal structures of MutS-DNA complexes from *E. coli* and human cells revealed that each MutS protein consists of two DNA-interacting domains, one ATP-interacting domain and two connecting domains [72, 73]. Most of the MutS-DNA interactions are with the DNA backbone and therefore not nucleotide specific, except for the previously mentioned phenylalanine and glutamate residues.

The ATP-binding cassette is an important factor contributing to mismatch recognition. When bound to perfectly matched DNA, ATP is quickly hydrolyzed by MutS, while binding to heteroduplex DNA inhibits ATP hydrolysis [74-76]. The ATP-bound dimer has greatly reduced affinity for homoduplex DNA, and only marginally reduced affinity for heteroduplex DNA [77]. Thus, the MutS-bound ATP complex is released from normal

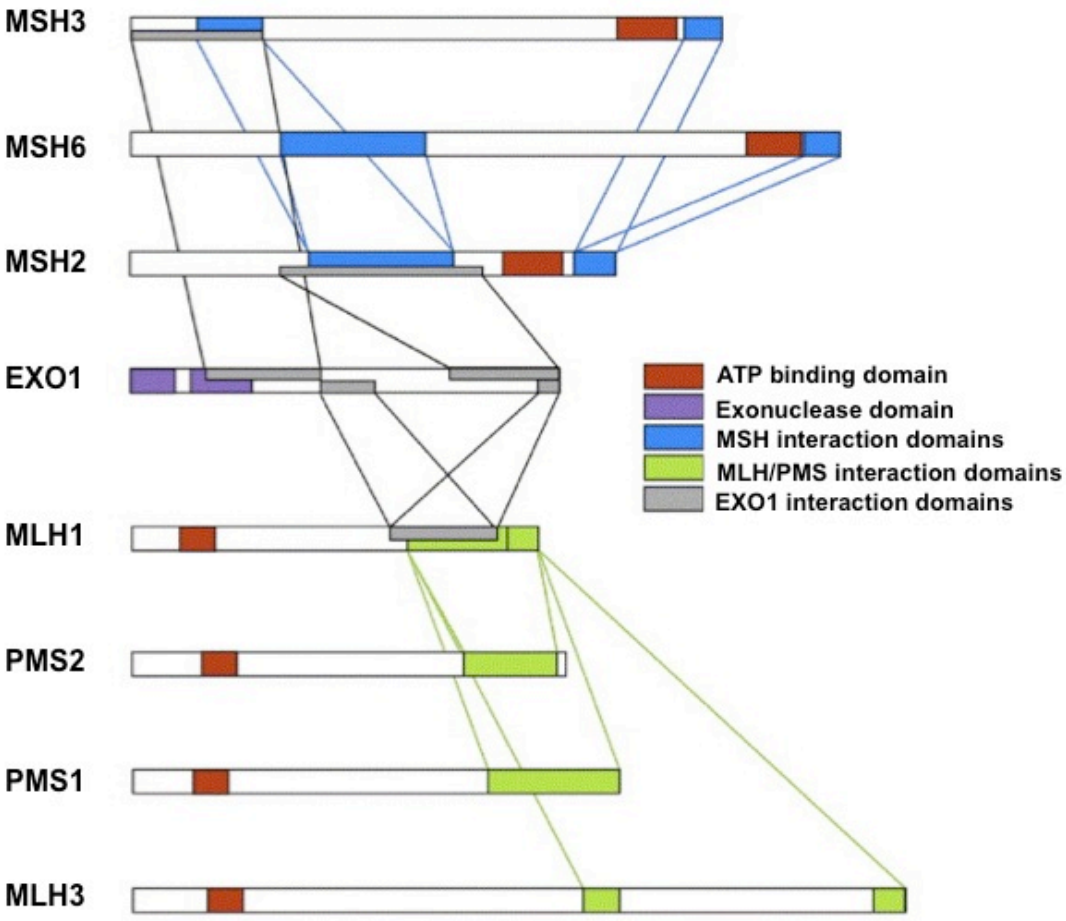


Fig 4. Interaction among the MMR proteins. Each of the MutS homologues (MSH3, MSH6, and MSH2) interacts as a heterodimer with the MutL homologues (MLH1, PMS2, PMS1, and MLH3), acting as heterodimers, and the exonuclease, EXOI. Mutations that occur in the interactive domains may abrogate the ability of these proteins to interact and function in DNA MMR, but, in some instances, the mutations may not lead to destabilization and loss of the protein product. The interaction domains among the MutS homologues, among the MutL homologues, and between one another are illustrated here. Adapted from [78].

DNA, but not from mismatch-containing DNA, which prolongues the lifetime of the MutS-DNA-ATP complex. This, together with its inhibited ATPase activity, stabilizes mismatch recognition and allows subsequent steps in mismatch repair.

The current mismatch recognition model [79, 80] suggests a DNA scanning activity for MutS. This is based on the observation that bent molecules are seen for both homo- and heteroduplex DNA [80]. Upon recognition of a mismatch, a conformational swich in the

protein causes kinking of the DNA. Finally, MutSα undergoes a second conformational change in which the DNA is unbent with the mismatched base possibly flipped out [81]. This final, unbending step, is perhaps a precaution before proceeding with the repair reaction.

3.2.2.4 Overall importance of mismatch repair and role in other metabolic pathways

Defects in mismatch repair predisposes to tumors of the colon, endometrium and other organs [82], and confers a mutator phenotype known as microsatellite instability (MSI) which is caused by the faulty repair of insertion/deletion loops during replication. One such MSI-displaying cancer is human-non-polyposis-colon-cancer (HNPCC), or Lynch syndrome, a cancer of the digestive tract. The first hint of this correlation was provided in the 1990s when germ-line mutations in MSH2 were observed in HNPCC families [83-86]). To date, mutations and epigenetic silencing in MSH2, MLH1, PMS1, MSH6 and PMS2 have been linked to this disease (although the latter three are rarely found) and account to 40-50% of these cancers [82, 86-88]. Germline mutations in MSH3 have not yet been identified in this context. As was expected from the known MMR functions, mouse knockout models for Msh2, Msh3, Msh6, Mlh1, Mlh3, Pms1, Pms2 and Exo1 [49, 89, 90] showed mice that were cancer-prone due to a mutator phenotype that became evident when analyzed for MSI. Table 2. summarizes these phenotypes.

Aside from its replicative function, mismatch repair proteins are also involved in other processes such as meiotic and mitotic recombination, immunoglobulin class-switching, somatic hypermutation, the stability of trinucleotide repeats, the repair of interstrand crosslinks and the DNA damage response [50, 83, 91-95].

Recent years have provided further insight into this intriguing pathway. Despite an evident methyl-directed strand discrimination signal in *E. coli* a parental-strand/daughter strand discriminating signal in eukaryotes remained a puzzle. Recently, an elevated incorporation of ribonucleotides (rNMPs) into the leading strand was reported [96, 97]. Biochemical work by this lab [98] provided evidence that repair of these rNMPs could act as entry points for MMR. This observation has sparked interest as to whether hijacking of other such lesions by the MMR machinery during replication could be the strand discrimination signal in eukaryotes.

Gene	MSI	Tumor	Fertility
MSH2	**YES**	**LYMPHOMA, G1, SKIN, OTHER TUMORS**	**YES**
MSH6	**YES**	**TUMOR FREE OR G1 TUMORS AT LATE AGE**	**YES**
MSH3	**LOW MSI IN DINUCLEOTIDE REPEATS**	**LYMPHOMA, G1 AND OTHER TUMORS**	**YES**
MLH1	**YES**	**LYMPHOMA, G1, SKIN, OTHER TUMORS**	**NO**
PMS1	**MONONUCLEOTIDE REPEATS ONLY**	**NONE**	**YES**
PMS2	**YES**	**LYMPHOMA AND SARCOMA**	**MALE INFERTILE**
MLH3	**YES**	**NOT AVAILABLE**	**NO**
EXO1	**YES**	**LYMPHOMA**	**NO**

Table 2. Phenotype of MMR deficient knockout-mice. Taken from [99].

Work linking MMR with epigenetic remodelling of chromatin has also surfaced in recent years. It appears that permissive chromatin is not only needed for transcription-related events, but also for MMR [100-103]. Also thought-provoking, a specific histone modification has been proposed to participate in recruitment of MMR to the chromatin during replication [104]. It is also likely that MMR proteins are themselves post-translationally modified. In fact, phosphorylation of MSH6 and MSH2 has been reported, even though its precise role is not well understood [105, 106]. Possible modification of both proteins by ADP-ribosylation, mainly MSH6, has also been observed *in vitro* [107, 108]. These observations add a further layer of complexity to all protein-protein interactions published regarding when, where and how these modifications could occur on MMR proteins and how they are linked to intact MMR function in cells.

3.2.3 Base excision repair

The base excision repair (BER) pathway removes damaged bases from our DNA that are generated by several endogenous and exogenous chemical alterations in the cellular environment [109]. It begins with recognition of the damaged base by a DNA glycosylase, of which there are 11 known in humans (see Table 3) [110, 111]. The glycosylase removes the damaged nitrogenous base by flipping it out of the DNA and cleaving the N-glycosidic bond. The DNA backbone remains intact, but this activity creates an apurinic/apyrimidinic (AP) site which is then targeted by an apurinic/apyrimidinic (AP) endonuclease such as (APE1). The phosphodiester bond is then cleaved 5' to the AP site, generating a nick or single-strand break (SSB) in the DNA. Monofunctional glycosylases lack lyase activity and depend on polymerase β to remove the 5'-deoxyribose phosphate and proceed to insert a new, undamaged nucleotide to the 3' end of the nick [112]. A bifunctional glycosylase has both glycosylase and AP lyase activity that removes the base and cleaves the phosphodiester bond 3' to the AP site. Repair is completed by the X-ray repair cross-complementing protein 1 (XRCC1)-DNA ligase IIIα complex [113, 114], which seals the nick. This would be a classical short-patch BER reaction.

A variation of this is long-patch BER [115], which occurs when processing of the damage by the glycosylase leads to SSBs that cannot be sealed. Polymerase β then inserts a nucleotide into the nick, and proteins such as polymerase δ, PCNA and RFC displace the strand further, creating a 2-12 nucleotide flap. Flap endonuclease 1 (FEN1) removes this structure and the final nick is sealed by DNA ligase I [116].

Human glycosylase	Type	Substrates
MPG	**monofunctional**	**3-meA, hypoxanthine**
UNG	**monofunctional**	**uracil**
OGG1	**bifunctional**	**8-oxoG, FapyG**
NTH1	**bifunctional**	**Tg, hoU, hoC, urea, FapyG**
NEIL1	**bifunctional**	**Tg, hoU, hoC, urea, FapyG, FapyA**
NEIL2	**bifunctional**	**AP site, hoU**
NEIL3	**bifunctional**	**unknown**
MYH	**monofunctional**	**A:8-oxoG**
SMUG1	**monofunctional**	**hoU, hmU, fU**
TDG	**monofunctional**	**T:G mispair**
MBD4	**monofunctional**	**T:G mispair**

Table 3. Overview of human DNA glycosylases. Glycosylases are listed as monofunctional or bifunctional. MPG, 3-methyladenine DNA glycosylase; UNG, uracil DNA glycosylase; OGG1, 8-oxoguanine glycosylase; NTH1; Neil1/2/3, Nei-like glycosylase; SMUG1, single-strand selective monofunctional uracil DNA glycosylase; TDG, Thymine DNA glycosylase; MBD4, methyl-CpG binding domain 4; 3-meA (3-methyladenine), 8-oxoG (8-oxoguanine), Tg (thymine glycol), hoU (5-hydroxyuracil), hoC (5-hydroxycytosine), FapyG/FapyA (formamidopyrimidine derivatives of guanine or adenine), hmU (5-hydroxymethyluracil), fU (5-formyluracil). Adapted from [111].

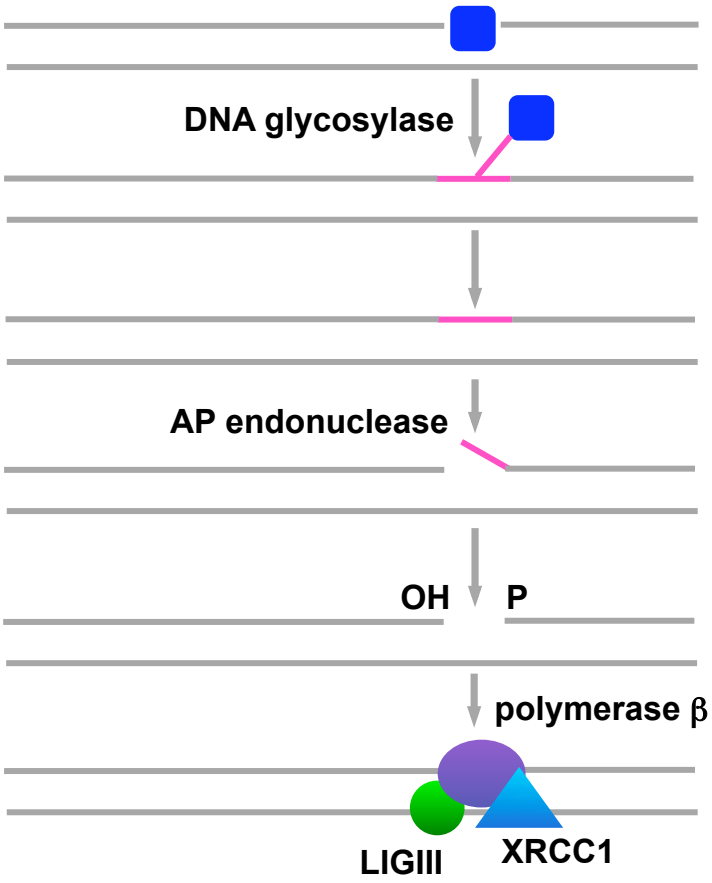


Fig. 5. Schematic overview of base excision repair (short-patch). The first step is the recognition of a damaged base (blue) by a DNA glycosylase. This enzyme flips the base out of the DNA and cleaves the N-glycosidic bond, creating an AP site (pink). The sugar-phosphate backbone is then cleaved by an AP endonuclease, which creates a nick in the DNA. Then DNA polymerase β fills in the gap with the correct nucleotide and the XRCC1/ligaseIIIα complex seals the nick. Adapted from [117].

3.2.4 Nucleotide excision repair

The term nucleotide excision repair (NER) is ascribed to a repair pathway that senses distortion in the DNA rather than a specific substrate. It is of vital importance, since it adresses adducts generated by dietary mutagens (nitrosamines, aflatoxin), through exposure to pollutants in the air (benzo(a)pyrenes) and, last but not least, sunlight (UV-induced cyclobutane pyrimidine dimers or (6-4)pyrimidine-pyrimidine photoproducts). NER also repairs lesions induced by crosslinking agents. These can either covalently bond DNA on the same strand (intrastrand crosslink) or across both strands (interstrand crosslink) (Nouspikel, 2009). The latter is repaired by a combination of NER together with other pathways, and it is recognized as a type of repair on its own, namely, interstrand crosslink repair (see 3.2.7). An important subset of NER, transcription-coupled repair (TCR), is coupled to transcription and was first documented in 1985. This study showed that removal of pyrimidine dimers (CPDs) was very efficient in an expressed gene [118]. RNA polymerase II stalled at a lesion is the sensor in this sub-pathway [119].

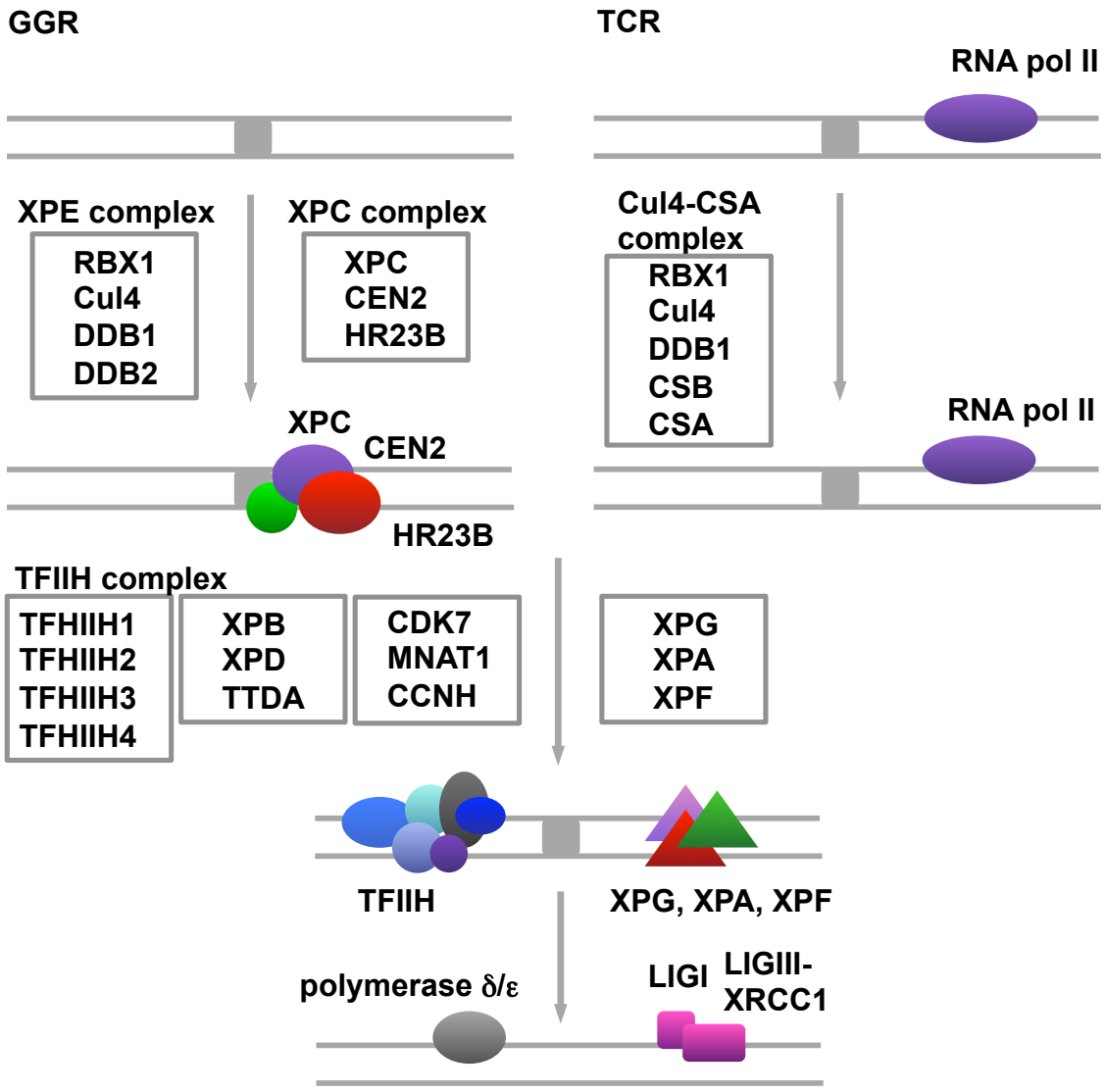


Fig. 6. Schematic overview of nucleotide excision repair (NER). Left, global genome repair (GGR) removes lesions such as UV-induced pyrimidine dimers throughout the genome. Lesions are recognized by the XPE and XPC complexes. Subsequently, other proteins are recruited to the site for further verification and dual incision by structure specific endonucleases XPG and XPF/ERCC1 enables the removal of the damage-containing oligonucleotide. The repair patch is then filled in and sealed by replicative polymerases and ligases I and III. Right, transcription-coupled repair (TCR), a sub-pathway that repairs actively-transcribed genes upon RNA polymerase II stalling. The two pathways converge after damage recognition. Here, recognition is mediated by the Cockayne syndrome group A (CSA) complex when the lesion stalls RNA polymerase II. Adapted from [120].

All other NER in the cell is known as global genome repair (GGR). The principal disease associated with defects in this repair pathway is *Xeroderma pigmentosum*, which can be caused by mutations in seven proteins of this pathway (XPA-XPG) and is characterized by sensitivity to sunlight accompanied by cancer predisposition [121]. In fact, the name of several NER players stems from their involvement in the disease. For example, the *Xeroderma pigmentosum* complementation group C protein is known as XPC.

Sensoring of the bulky DNA lesion is pivotal in this pathway and is the first repair step [122] (see Fig. 6). Distorted, chemically-modified DNA can be recognized by XPC/HR23B/Centrin2 [123]. Of the three, XPC is believed to perceive the distortion [124], Centrin2 improves its efficiency and HR23B might play a role in XPC ubiquitination [125], which in turn, increases affinity for the damaged DNA. Another complex involved in lesion recognition is the DNA damage binding protein (DDB) complex, a heterodimer consisting of DDB1 and DDB2(XPE) [126]. This particular complex can exist as a component of a Cul4/RBX1-based multisubunit E3 ubiquitin ligase not only for GGR, but also within TCR. In this repair scenario, however, it acts within a Cockayne syndrome group A (CSA)/CSB complex [127, 128]. A striking difference of the two paths of recognition is that in TCR, a substrate, CSB, is targeted for degradation via ubiquitination, whereas in the other, the ubiquitin ligase activity leads to XPC polyubiquitination but not to its degradation [129]. The role of the TFIIH complex, which is comprised of 10 subunits (ERCC2(XPD), ERCC3(XPB), p62(TFIIH1), p52(TFIIH4), p44(TFIIH2), p34(TFIIH3), TTDA, CDK7, MAT1, and Cyclin H), is to ensure the formation of a denaturation bubble spanning the lesion [130]. Althought the precise function of all of these subunits is not known, the XPD and XPB helicases play an important function in opening up the DNA [131], although in the case of XPB it is its ATPase, rather that its helicase function, which is required [132].

A further gap in knowledge is provided by the involvement of XPA [133]. It is associated with RPA [134], absolutely required for the repair and it might aid in discriminating the lesion-containing strand from the intact DNA strand, but this possibility has not been conclusively explored. The next step requires incision on either side of the denatured bubble so that a damage-containing fragment of 25-30 nucleotides in length can be excised. XPG cuts 3' to the lesion and XPF/ERCC1 5' to the lesion [135, 136]. The penultimate step requires filling of the generated gap by replicative DNA polymerases

such as pol δ and ε (Popanda & Thielmann, 1992). The repair is completed upon ligation of the nicked DNA. Previously, it was believed that ligase I carried out this task. More recent data identified ligase III together with its partner XRCC1 as the main ligase involved [137].

3.2.5 Double-strand break repair

Double-strand breaks (DSBs) are breaks in DNA that arise throughout the cell cycle and can be successfully repaired in error-prone as well as error-free ways. They are generated upon cell irradiation (γ radiation and X-rays) for example, or treatment with clastogenic agents, and are also features of replication [138]. Repair of unwanted double-strand breaks is distinct from the metabolism of programmed DSBs that are required for e.g. lymphocyte development [139], meiotic recombination [140], or pathways that mask structures that could resemble DSBs, such as telomere ends [141]. Two main pathways of this repair type can be recognized: non-homologous end-joining (NHEJ) and homologous recombination (HR) (see Fig. 7).

3.2.5.1 Non-homologous end joining

NHEJ can take place throughout the cell cycle, whereas HR can only occur after genomic replication when a sister chromatid guides DSB repair [142]. As its name implies, NHEJ relies on direct ligation of the broken ends, which is quite inaccurate since it can process altered or improper ends. Another form of NHEJ is micro-homology mediated end joining (MMEJ), which uses small homologous regions on the two sides of the DSB to align the broken strands before joining [143]. NHEJ is an error-prone mode of repair, since sequence information is lost . NHEJ is associated with local activation of the DNA dependent protein kinase (DNA-PK) which is triggered by the binding of the broken ends by the Ku70/Ku80 heterodimer [144]. Often, processing of the ends is required before Ku binding. This can be mediated by the protein Artemis for example, a 5'-3' exonuclease which acquires endonuclease activity upon phosphorylation by DNA-PK and relieves the formation of hairpins within the two strands of the DSB [145]. End-ligation is performed by DNA ligase IV, together with X-ray cross-complementation group 4 (XRCC4) and XRCC4-like factor (XLF) [146, 147].

3.2.5.2 Homologous recombination

In HR, 5'-3' resection of the broken ends creates two single-stranded DNA regions. This is mediated by the MRN complex meiotic recombination 11 homolog (Mre11)/Nijmegen breakage syndrome 1 (Nbs1)/Rad50, Exo1, Dna2, CtBP-interacting protein (CtIP) and possibly the Bloom (BLM) helicase [148-151]. This, in turn, leads to recruitment and activation of ATM (see 3.4.1). ATM phosphorylates a set of substrates including H2AX, creating the DSB marker γH2AX [152]. This phosphorylation signal helps amplify a ubiquitination signal in response to DSB and other proteins, for example 53BP1 and breast cancer 1 (BRCA1) are recruited to the site [153]. The free 3' ends are then coated by RPA, which leads to parallel activation of the ATR kinase in response to DSBs [154]. RPA is then displaced by Rad51 and associated factors to form so-called synaptic filaments in a BRCA2-mediated manner and this filament can then invade a homologous neighbouring sister chromatid and perform a homology search [155, 156]. This creates a specific structure, a D-loop, as well as an X-shaped structure called a Holliday junction (HJ) [157] and synthesis from the invading 3' strand can occur. *In vitro* studies have implicated pol η in this extension [158].

From this point onwards several possibilites are available, depending on the type of break and proteins involved. The most prevalent repair type is synthesis-dependent strand annealing (SDSA) where regulator of telomere length 1 (RTEL1) reverses the D-loop and thus prevents an exchange of genetic material beween the homologous chromosomes (non-crossover repair). Conversely, realignment or capture of the newly-synthesized end forms a double Holliday junction (HJ) [159] structure that can be adressed via crossover or non-crossover operations. A complex formed by the Bloom helicase (BLM) and TOPOIIIα can dissolve this structure into a non-crossover, the structure specific Mus81/EME1 endonuclease can resolve this structure into a crossover and HJ resolvases such as GEN1 can resolve the structure in ways that can result in a crossover or not [160-162]. Finally, it should be mentioned the HR has a role in replication fork restart after stalling due to interstrand crosslinks, for example.

When it comes to pathway choice, not only do CDKs play an important role in asserting activity of pathway players during the cell cycle [163], but this year, a report focusing on 53BP1 and BRCA1 has shed further light into this matter. These two proteins are seemingly antagonistic: 53BP1 promotes NHEJ and BRCA1 promotes HR, yet, they are

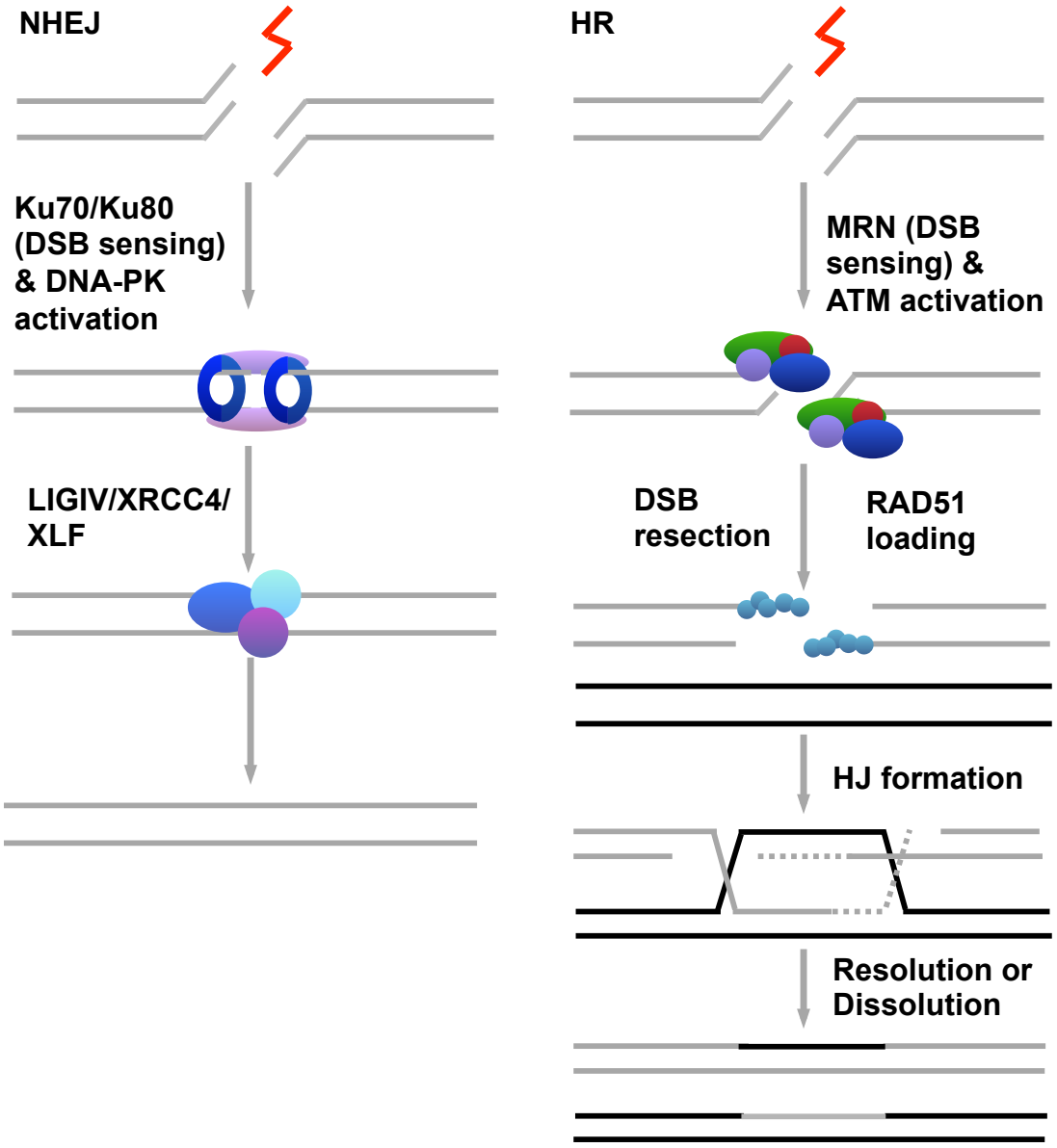


Fig. 7. Schematic overview of the two main double-strand break repair pathways, non-homologous end joining (NHEJ) and homologous recombination (HR). Left, NHEJ pathway. The Ku70/Ku80 heterodimer senses the double-strand break (red), stabilises the two ends and recruits DNA-PK. DNA-PK phosphorylates and activates a complex consisting of ligase IV, XRCC4 and XLF, which religates the broken DNA. Right, HR pathway. The MRN complex (Mre11/Rad50/Nbs1) recruits ATM to the damaged site. This leads to ATM activation and phosphorylation of multiple substrates. The broken ends are resected by the action of proteins such as Mre11, CtIP and Exo1, and Rad51 coats the ends and then nucleoprotein filaments then invade the undamaged sister strand forming Holliday junction (HJ) structures. DNA synthesis and the activity of HJ resolvases, structure specific endonucleases or helicases proceed to complete the repair. Adapted from [164].

both recruited to DSBs. It was unclear how their accumulation at DSB could set up pathway choice. It was reported by the Durocher group that in G1, even though expressed, BRCA1 does not form foci due to the recruitment of the Rap1 interacting factor 1 (RIF1) to the DSB. This recruitment is somehow mediated by the phosphorylation of 53BP1 by ATM. Alternatively, accumulation of RIF1 at DSBs during S ahd G2 phases can be inhibited by BRCA1 and CtIP, thus promoting HR. Remarkably, depletion of RIF1 can restore DSB repair to resection and Rad51 loading in BRCA1-depleted cells [165].

3.2.6 DNA damage bypass

DNA damage bypass refers to a tolerance mechanism evolved to aid genome replication, albeit at the expense of genome instability by using error-prone translesion synthesis (TLS) polymerases that allow circumventing lesions instead of stalling and/or slowing down of replication forks. TLS polymerases have a more flexible catalytic pocket that allows replication across and past damaged templates at the expense of processivity. Examples of TLS polymerases (see Table 4) include pol λ, pol η, pol ι, pol κ, pol ζ and REV1 (the latter is believed to act as a scaffold in recruiting other TLS polymerases and is a deoxycytidyl transferase rather than a polymerase) [166].

Bypass of lesions in this manner is largely regulated by two small proteins, ubiquitin (76 amino acids) and SUMOs 1-4 (ca. 100 amino acids). At the centre of this is PCNA, which acts as a molecular switch for post-translational modification with these proteins mainly via its K164 residue. Both ubiquitination and SUMOylation require an E1 conjugating enzyme, an E2-activating enzyme and an E3 ligase to modify PCNA, either once (e.g monoubiquitinated PCNA, SUMOylated PCNA) or multiple times (e.g polyubiquitinated PCNA). The Rad6 E2 ubiquitin conjugator and the Rad18 E3 ubiquitin ligase take over monoubiquitination of PCNA both in yeast and humans [167]. Rad18 binds single-stranded DNA and could thus contribute to the recognition of the damage. Polyubiquitination of PCNA requires the yeast E2 Ubcl3-Mms2 and E3 Rad5 ligase but the precise trigger for this signal remains unknown [168]. DNA damage-dependent monoubiquitinated PCNA supports an error-inducible pathway involving TLS polymerase recruitment, whereas polyubiquitinated PCNA is believed to lead to error-free template switching. In general, polymerase stalling or exposure of single-stranded DNA is considered the trigger for activation of the TLS pathway. Many of these TLS polymerase

have been shown to interact directly with PCNA or monoubiquitinated PCNA. For example, pol η has a higher affinity for monoubiquitinated PCNA, which would help target it to damage where it would then bypass thymine photodimers [169, 170]. The precise role of PCNA SUMOylation on the other hand, remains obscure. It might increase the activity of an antirecombinogenic protein, yeast Srs2, to avoid HR and

TLS polymerases	Function
η (eta)	Bypass UV lesions
ι(iota)	Bypass synthesis
κ (kappa)	Bypass synthesis
λ (lambda)	BER, NHEJ
μ (mu)	NHEJ
θ (theta)	DNA repair
ζ (zeta)	Bypass synthesis
Rev1	Abasic sites
ν (nu)	Unknown

Table 4. Overview of human TLS polymerases.

favor bypass. What complicates this scenario is that a human homologue has not yet been identified so corroborating this hypothesis will be difficult. Adding salt to injury, detection of PCNA SUMOylation in human cells, probably due to its transient nature and subtle targetting of PCNA, had not been reported until recently [171, 172]. Nevertheless, it should be noted that there are human proteins such as ELG1 that contain SUMO-interacting domains [173]. This protein is being studied with regard to its possible link to replication and participation in HR. Thus, a role of PCNA SUMOylation in human cells should not be ruled out [174].

3.2.7 Interstrand crosslink repair

DNA interstrand crosslinks (ICLs) are lesions that covalently link two DNA strands. They are extremely cytotoxic, since they present a block for both replication and transcription. They are repaired by a combination of different repair pathways, in which the *Fanconi anemia* (FA) pathway takes centre stage (see Fig. 7).

At least 15 FA gene products are known to be involved in key steps of the repair. Stalling of the replication fork upon encountering the ICL is recognized by the FA complementation group M (FANCM)/FA associated protein of 24 kDa (FAAP24) complex, which also participates in the activation of the ATR pathway. ATR, which is recruited to RPA-coated single-stranded DNA in the stalled replication fork, can then readily phosphorylate target FA proteins belonging to the core complex (FANCA, B, C, E, F, G, L, M) and the FANCI/FANCD2 complex [175, 176].

Monoubiquitination of FANCD2 and FANCI is crucial in fueling clear activation of the pathway and serves as a recognition marker for interstrand crosslink repair. The E3 ubiquitin ligase responsible is the FANCL FA core subunit, together with the E2 ubiquitin conjugator UBE2T [177, 178]. The monoubiquitinated heterodimer is relocalized to the lesion where it coordinates downstream repair events such as the recruitment of nucleases like FA-associated nuclease I (FAN1), FANCP(SLX4) , MUS81/EME1 and XPF/ERCC1 that can create incisions on one or both sides of the ICL to allow unhooking of the lesion [179-181]. The unhooking of the lesion turns a collapsed replication fork into a double-strand break (DSB). To overcome this, translesion DNA synthesis (TLS) proteins are recruited to the lesion in a manner that remains unclear, and this allows bypass of the unhooked crosslinked oligonucleotides and restoration of a nascent strand. The homologous recombination (HR) pathway with the aid of downstream FA proteins such as FANCD1/BRCA2, FANCJ, FANCN and FANCO, repairs the DSB and NER excises the remaining unhooked oligonucleotide. The gap which is left behind is filled in by DNA polymerases.

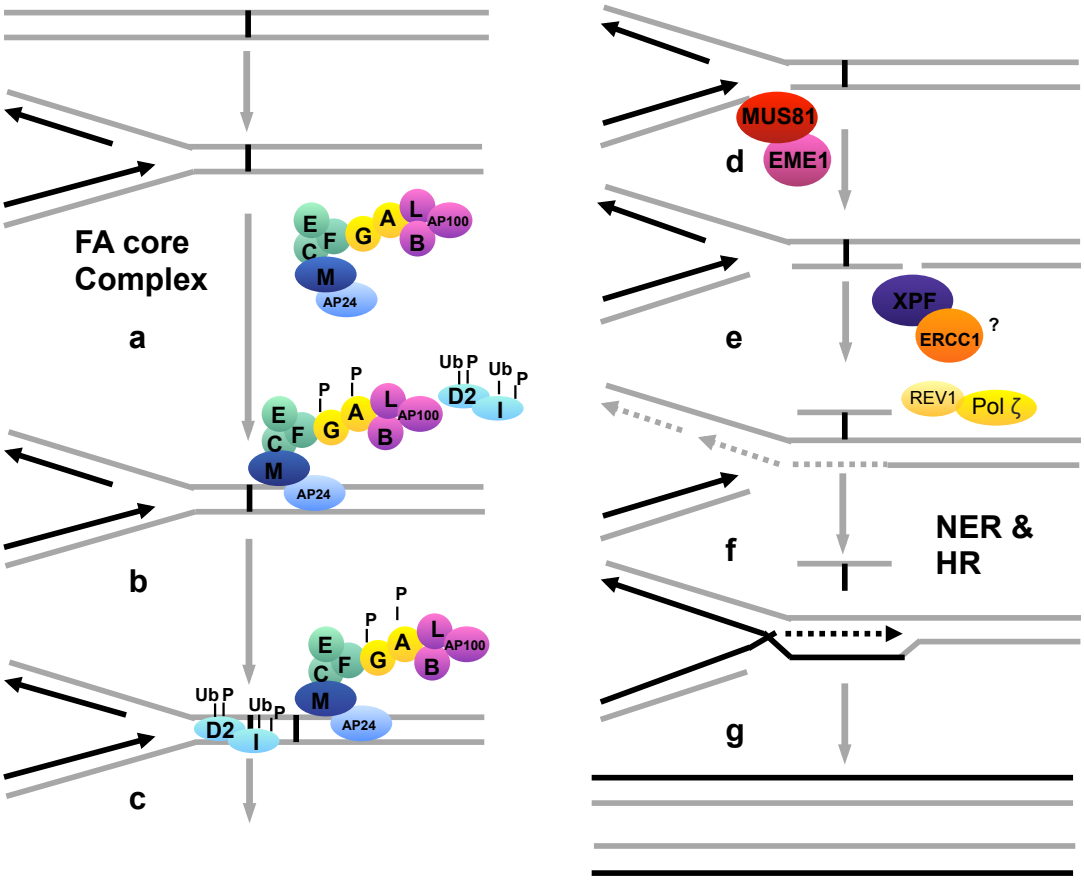


Fig. 7. Schematic representation of interstrand crosslink repair. Replication fork stalling upon encountering an interstrand crosslink leads to phosphorylation of several subunits of the Fanconi anemia (FA) core complex (FANCA, B, C, E, F, G) by ATR and recruitment to the lesion by FANCM/FAAP24. The E3 ubiquitin ligase FANCL (a) monoubiquitylates the FANCD2/FANCI heterodimer (b), MUS81 cleaves the lesion on one side and XPF/ERCC1 unhooks it on the other (c and d). Translesion polymerases then fill the gap opposite the unhooked nucleotide (e), which is released by excision repair (f). HR then rescues the collapsed fork with the intervention of several proteins (e.g. BRCA2, FANCN, likely also FANCJ (f and g)). Adapted from [179].

Mutations in the FA proteins or disruption of the repair process by, for example, failing to deubiquitinate the FANCD2/FANCI complex leads to hypersensitivity to crosslinking agents in cells [182]. Indeed, the name for the FA proteins stems from their association with the inherited disease *Fanconi anemia* (FA), a chromosomal instability disorder characterized by bone marrow failure, congenital defects and a predisposition to certain types of cancer [183, 184].

The study of this type of repair extends to ICLs occuring outside of replication, and is refered to as replication-independent ICL repair. Components of this pathway include NER proteins and translesion polymerases such as pol κ and exclude FA proteins or Rad51, which is imperative for formation of homologous HR intermediates during the replication-coupled repair [185, 186].

3.3 Mismatch repair lead DNA damage tolerance and clinical implications

As previously mentioned, MMR proteins are implicated in HNPCC. It is not clear how these mutations arise and what precise molecular consequences play out in these patients, but the lack of MMR surveillance during DNA replication is most likely the root cause of the genomic instability observed. Ironically, MMR functions are not only required for stability that would prevent disease onset, but they are used in a clinical setting to treat certain diseases. This is due to the fact that there is a clear link between MMR and drug resistance.

For example, both BER and MMR can remove 5-fluorouracil (FU) from DNA, a drug that is used in treatment of colon cancer [187]. MutSβ was recently described to participate in this recognition [188]. A MMR-dependent two-fold resistance to cisplatin can also be observed. It appears that cells treated with this agent accumulate defects in MMR that help them circumvent its toxicity [189-191]. The main adduct generated in this case is a 1,2-intrastrand crosslink between the N7 atoms of two adjacent purines. This adduct is normally repaired by NER, but it was shown *in vitro* that MutSα could bind this type of crosslink [192]. Treatment with this agent induces a rapid arrest during S phase, since it presents a block for replicative polymerases and this in turn activates many stress-response pathways. This response could involve, in part, an interaction between MMR and a transcription factor during apoptosis in these cells [193]. In accordance with this, induction of MSH2 expression by the p53 transcription factor might take over the repair of pyrimidine photodimers cause by exposure to sunlight [194] and contribute to resistance to UV damage [195]. Studies in yeast support the notion that there might be crosstalk between NER and MMR proteins [196].

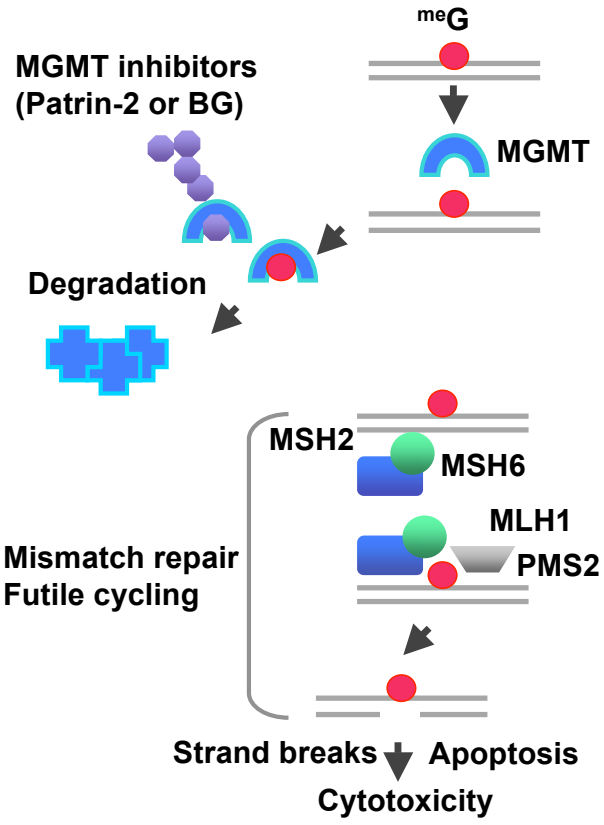


Figure 8. Role of MMR in the response to alkylators. Temozolomide and other S_N1-type alkylators generate the toxic lesion O6-methylguanine (^{me}G, red circle). MGMT (O6-methylguanine DNA methyltransferase) normally repairs this toxic adduct by direct removal of the methyl group in a suicide reaction. Inhibitors such as Patrin-2 and O6-benzylguanine (BG) lead to MGMT degradation. The MMR machinery, which includes the MutSα (MSH2/MSH6) and MutLα (MLH1/PMS2) heterodimers then takes over and elicits cell death during a second cell cycle. Adapted from [197].

More problematic seems to be the response to agents such as 1-(2-chloroethyl)-e-cyclohexyl-nitrosourea (CCNU), which generates interstrand crosslinks. A body of data report cell sensitivity towards treatment with this agent in a MMR-deficient background [198-200]. Since this is the only agent in which MMR defects contribute to sensitivity and not resistance this is certainly noteworthy. Since this sensitivity seems to involve MutSβ ρecognition of the lesions [201, 202] more studies are neccessary to clarify this scenario.

At the opposite end of the spectrum, the most corroborated and striking MMR-associated tolerance to an agent is the 100-fold resistance observed towards S_N1-type alkylators (temozolomide, MNNG, MNU) in MMR deficient cells. This is of relevance in tumors where O6-methylguanine-methyltransferase (MGMT) activity has been inhibited, a therapy approach used in the treatment of certain types of melanoma, leukemia and glioblastoma [203, 204]. It is generally accepted that O6-methylguanine or ^{me}G is the primary cytotoxic lesion, since its presence in the DNA leads to the formation of $^{me}G/C$

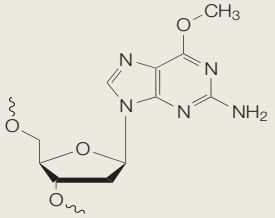
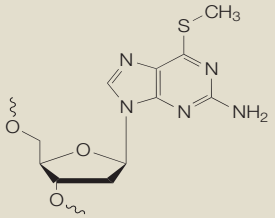
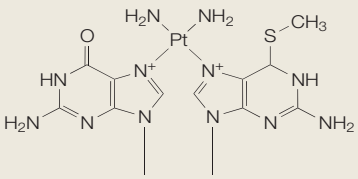
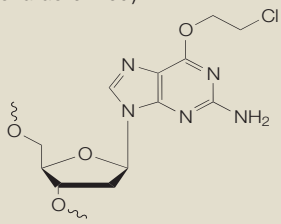
Substance type	Substance	Cytotoxic metabolite	Mode of action
S_N1-type methylating agents	*N*-methyl-*N*-nitrosourea (MNU), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), temozolomide, procarbazine, dacarbazine, streptozotocin	6-*O*-methyl-2′-deoxyguanosine 	^{Me}G base pairs with T during replication. These lesions are unsuccessfully addressed by the MMR system, which results in a cell-cycle arrest in G2
Antimetabolites	6-Thioguanine	6-*S*-methylthio-2′-deoxyguanosine 	As above, but two replication cycles are required, because 6-thioguanine has to be incorporated into DNA before being methylated
Intrastrand crosslinking agents	*Cis*-diammine-dichloroplatinum (II) (CDDP; also known as cisplatin)		This lesion is repaired primarily by the NER pathway, but if it persists until replication, it would arrest the replication fork, involving MMR proteins in the process
Intrastrand crosslinking agents	1-(2-chloroethyl)-3-cyclohexyl- nitrosourea (lomustine, CCNU), mitomycin-C (MMC)	(DNA adducts of MMC are not well characterized) 	The chloroethyl group can react with the opposite DNA strand to generate a covalent crosslink. Some MMR-deficient cells are more sensitive to these substances than MMR-proficient ones. The mechanism of MMR involvement is unknown

Table 5. Substances with cytotoxicities that are related to MMR status. Taken from [54].

and ^{me}G/T mispairs that activate MMR [205] but are irreparable because the ^{me}G is in the template strand during replication. This leads to a delayed arrest during the second cell cycle that is mainly ATR-dependent [206, 207] (see Fig. 8). Interestingly, the homologous recombination machinery is required for the damage tolerance during the first S phase [208]. This gave rise to speculation as to the nature of the intermediates formed upon MMR processing [209]. Treatment with yet another anti-cancer drug, 6-thioguanine, is also partially dependent on MMR status. Only a 10-fold resistance to it can be seen in MMR-deficient cells, however [210-213].

3.4 Signaling networks in response to DNA damage

That mismatch repair responds to treatment with various chemotherapeutic and similarly-acting agents is clear, but what is the nature of the signaling involved? Which main pathways elicit MMR-mediated toxicity and what are they activated by?

A coordinated signaling response to several types of DNA damage is elicited by two main kinases which belong to the PIKK (phosphatidylinositol-3-kinase related kinases) [214] family: ATM (Ataxia telangiectasia mutated) and ATR (Ataxia telangiectasia and Rad3 related). A further PIKK member, DNA-PK, is known to stimulate repair activities locally, but without triggering a widespread cellular response. Activation and signal transduction by these two main players is mainly associated with phosphorylation events, although recent years have underscored the merits of SUMOylation and ubiquitylation in these events as well [215]. Members of the PIKK family (see Fig. 9) are large proteins (>300 kDa) with the following structures (see Fig. 9): a variable number of repeat domains at the N-terminus (HEAT domains), a FAT domain, a catalytic domain homologous to that of PI-3 kinase, a PIKK regulatory domain (PRD) and a FATC domain at the very C-terminus [216].

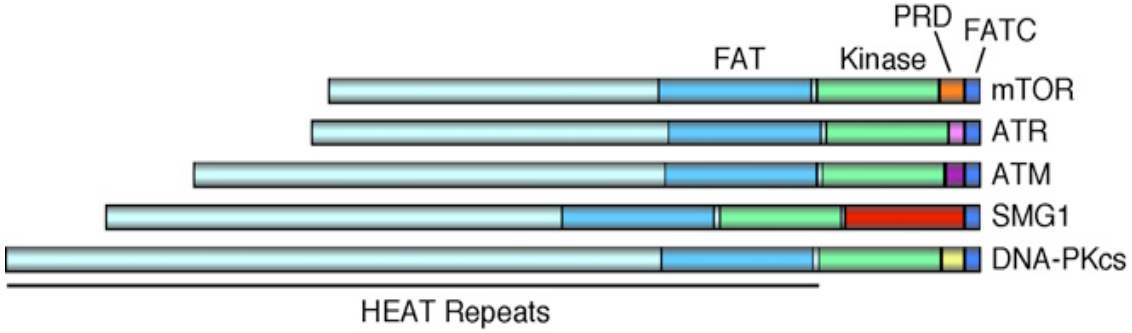


Fig. 9. Diagram of PIKK domain structures. Taken from [216].

3.4.1 ATM and ATR

As mentioned previously, treatment of MMR-proficient cells with S_N1-type alkylators triggers a G2/M arrest after two cell cycles that is intrinsically linked to the ATR kinase.
Within DNA damage response networks, ATM and ATR are key regulators in vertebrates. They associate with DNA and preferentially phosphorylate substrates at S/T-Q (serine/threonine/glutamate) residues [217-219]. In general, ATM is activated throughout the cell cycle in response to numerous DSBs whereas ATR is activated by

ssDNA tracts formed during the S and G2 phases of the cell cycle. Like its name indicates, ATM is perhaps most recognized in conjunction with the hereditary disorder Ataxia-Telangiectasia [220]. This is a severe autosomal recessive disease characterized by early onset lack of voluntary coordination of muscle reflexes (cerebellar ataxia), small dilated blood vessels on the skin surface (telangiectasia), immunodeficiency and predisposition to lymphomas.

In a way that is not completely understood, ATM activation is triggered by the recruitment of the MRN (MRE11/Rad50/Nbs1) complex to DSBs, caused by ionizing radiation or treatment with campthotecin, for example [221-223]. It is thought that ATM is an inactive homodimer that dissasociates and activates in response to damage, probably due to its autophosphorylation at serine residue 1981. This remains controversial, as it has also been shown that its autophosphorylation is not required for its activation *in vivo* [224-226]. ATM monomers are then recruited to DSBs via interactions with the MRN sensor complex, which provides a platform for local ATM substrate phosphorylation [227]. These substrates include the histone variant H2AX, which forms the γH2AX mark [228], the MRN complex itself, SMC1 [229] and its downstream effector Chk2 [230]. ATM phosphorylates Chk2 at a specific threonine, T68, which leads to homodimerization and full activation of this kinase [231-233]. Once activated, Chk2 monomers disperse [234] and act on multiple downstream targets such as p53 [235] and Cdc25 family phosphatases [236], 53BP1 [224] and the BRCA1 tumor suppressor [237], amongst others.

In contrast to ATM, ATR is essential for cell survival. ATR and Chk1 (ATR downstream target) knockout mice are embryonic lethal [238, 239]. Known ATR-related diseases include the rare Seckel syndrome, whose symptoms include mental retardation and microcephaly. Patients with this disease live with very low amount of ATR protein [240]. Activation of ATR can be traced to several sources of DNA stress such as replication fork stalling, nucleotide depletion, DSB resection, etc [241]. What is ultimately recognized by the ATR machinery and activates it, is the appearance of ssDNA regions in the DNA. The molecular mechanism of ATR activation is well understood: ATR and ATRIP, its interaction partner, recognize ssDNA regions coated by RPA [242]. Rad17 and the 9-1-1 (Rad9-Hus1-Rad1) complex are independently recruited to the chromatin by the same structure [243]. This, in turn, is required for TopBP1 (Topoisomerase binding protein 1)

recruitment to these sites, where it acts as an ATR activator [244, 245]. Recently, the proteins Timeless and Tipin (Timeless-interacting protein) were implicated in ATR-Chk1 activation [246]. Another mediator is Claspin, which is also shown to associate with ssDNA and mediate activation of ATR and downstream phosphorylation of Chk1 [247, 248]. ATR phosphorylates Chk1 within the C-terminal regulatory domain, primarily on serine 345 (S345), although phosphorylation on serine 317 (S317) can also be observed. Activated Chk1 substrates include positive and negative regulators of Cdk inhibitory phosphorylation such as Cdc25A [249] and Cdc25C [250]. In addition to the DNA damage response, ATR functions in both S and M cell cycle phases to monitor replication origin firing, elongation, replication fork restart and centrosome instability [251-253].

Although ATM and ATR have distinct substrate specificities, it is becoming increasingly clear that these two pathways are interconnected and complementary [254]. In 2006 it was unveiled that ATM-dependent processing of DSBs, which is mediated by Mre11, forms ssDNA regions that then activate ATR [163]. Further studies have also provided support for the dual rual of ATM and ATR in DSB repair [154, 255]. Importantly, it was also shown that ATR can activate ATM in response to ssDNA [256].

3.4.2 DNA damage checkpoints

All three kinases involved in the DNA damage response are essential for DNA damage checkpoints throughout the cell cycle (see Fig. 10). Activation of these checkpoints in response to damage is neccessary to grant the cell more time for repairing damage.
Progression though different stages of the cell cycle is tightly regulated by cyclin-dependent kinases or CDKs. These are activated by cyclins and inhibited by CDK inhibitors or inhibitory tyrosine phosphorylations [257]. CDK1 is considered a mitotic CDK, whereas CDK2, 4 and 6 are active during interphase. Cell transduction pathways, like the ATM and ATR pathways, can limit the activity of CDKs and prevent cell cycle progression into the next stage (G1 and G2/M checkpoints) or also slow down cell cycle progression (intra-S checkpoint). More specifically, the G1/S checkpoint prevents replication of damaged DNA and the G2/M checkpoint provides surveillance before proceeding into chromosome segregation [217]. ATR and ATM, via activated Chk1 or Chk2, can induce the phosphorylation of the CDK1 and CDK2 inhibitor CDC25a during S phase, for example, which leads to the degradation of the Cyclin E/CDK2 complex. This

effectively hampers replication initiation [258]. Another example of this regulation involves the phosphorylation of p53 by ATM, ATR, Chk1 and Chk2. Many targets of this transcription factor are involved in cell cycle progression. An important target is the cyclin-dependent kinase inhibitor (CKI) $p21^{CIP1}$ which is involved in the G1/S checkpoint by inhibiting CDK2 and CDK4. Likewise, phosphorylation of Cdc25c by Chk1 or Chk2 prevents mitotic entry of damaged cells. This occurs because Cdc25c phosphorylation prevents it from targeting its substrate CDk1/CDC2-Cyclin B for dephosphorylation, thus preventing mitotic entry.

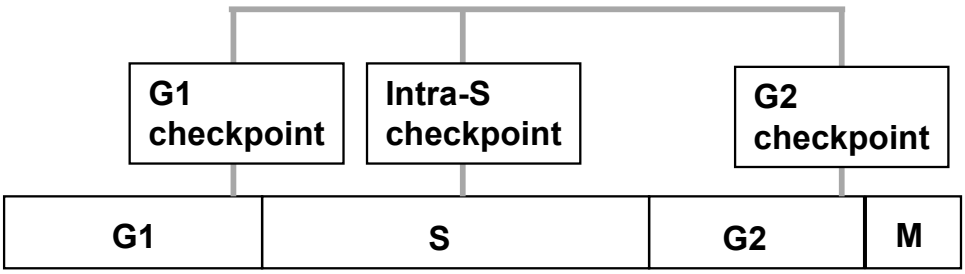


Fig. 10. Checkpoint responses in cells. DNA damage that delays entry in S phase causes a G1 checkpoint. An intra-S checkpoint occurs when replication is slowed down and replication forks need to be stabilised and/or suppressed until the damage is overcome and the G2 checkpoint prevents entry into mitosis when damage is persistent.

This part of the thesis ends with an overview of DNA repair protein defects associated with cancer.

Protein	Syndrome	Primary cancers	Biomarker
HR			
BRCA1, BRCA2	–	Breast, ovarian[112, 135]	–
RAD54B	–	NHL, colon cancer[113]	–
RAD51B (RAD51L1)	–	Lipoma, uterine leiomyoma[114]	–
CtIP (RBBP8)	–	Colorectal cancer[115]	–
NHEJ			
MRE11	Ataxia telangiectasia-like disorder	Colorectal cancer[108]	IR sensitivity
LIG4	LIG4	Leukaemia[116]	IR sensitivity
Artemis (DCLRE1C)	Omenn	Lymphoma[117]	–
MMR			
MSH2, MLH1, MSH6, PMS1, PMS2, MLH3	–	Hereditary nonpolyposis colorectal cancer[118–120]	Microsatellite instability
RecQ			
BLM	Bloom	Various[121]	Increased SCE
WRN	Werner	Various[122]	Increased telomeric SCE
RECQL4	Rothmund–Thomson	Skin basal and sqamous cell, osteosarcoma[122]	–
Damage signalling			
ATM	Ataxia-telangiectasia	Leukaemia[123]	IR sensitivity
NBS1	Nijmegen breakage	Various[126]	IR sensitivity
p53	Li–Fraumeni	Various[127]	–
CHK2	Li–Fraumeni	Various[128]	–
NER			
XPA, XPC, DDB1, ERCC4, ERCC5, POLH	XP	Skin cancers[129]	UV sensitivity
ERCC2, ERCC3	XP, Cockayne, trichothio-dystrophy	Skin cancers[129]	–
ERCC1	Cerebro-oculo-facio-skeletal	Squamous cell carcinoma, head and neck[130]	–
FA			
FANCA, FANCC, FANCD2, FANCE, FANCG	Fanconi anaemia	Various[131]	Impaired FANCD2 ubiqutination[131]
FANCB, FANCF, FANCL	Fanconi anaemia	Various[131]	Impaired FANCD2 ubiqutination[131]
BRIP1	Fanconi anaemia	Various[131]	–
FANCM	Fanconi anaemia	Various[131]	Impaired FANCD2 ubiqutination[131]
BER			
POLB	–	Various[132]	–
FEN1	–	Various[133]	–

Table 6. DNA repair genes and checkpoint genes implicated in cancer by pathway.

3.5 The *Xenopus laevis* egg extract system as a model for replication and replication-tethered DNA damage responses

South African clawed toad (*Xenopus laevis*) egg extracts have proven to be an indispensable tool for gaining mechanistic insights into DNA replication and the molecular machinery required for maintenance of our genome during S phase. Their potential as a model for replication was first recognized in a series of publications in the 1980s, which started with the observation that simian virus (SV40) chromatin could be replicated in protein extracts from *Xenopus laevis* eggs [259]. The same laboratory reported the use of single-stranded M13-DNA as a replicative template in this *in vitro* system a while later [260]. Meanwhile, Lohka and Masui [261, 262] showed that low-speed supernatants made from activated eggs induce chromatin decondensation and nuclear envelope formation in demembranated sperm nuclei and in 1986 it was demonstrated that these extracts can complete a single round of semiconservative DNA replication of sperm nuclei and plasmid DNA [263]. What followed was a multitude of publications investigating not only the sequence of events in replication initiation and elongation with the aid of this tool, but also providing novel insights into known replicative proteins as well as identifying new replication factors. Almost concomitantly, DNA repair of damaged DNA templates in the extracts was described [264-266].

But what characteristics do these eggs possess that make them so useful in biochemical experiments?

First and foremost, *Xenopus* proteins show a good homology with their human counterparts. Secondly, mature *Xenopus laevis* oocytes are large cells. Their diameter is about 10^6 times that of a somatic cell and the protein content of a single oocyte is the equivalent of 25 μg of cytoplasmic protein. They are arrested in metaphase of meiosis II by cytostatic factor (CSF), which is responsible for maintaining high intra-cellular levels of Cdk1/Cyclin B, ready for ovulation and fertilisation [267].

When fertilisation occurs, an increase in calcium channels the degradation of CSF and Cyclin B, which releases the G2-arrest so that the egg can transit from metaphase, through anaphase, to interphase. The fusion of male and female pronuclei paves the way for replication of the zygotic genome, mitosis and 11 subsequent and alternating S and M phases of the cell cycle. Following this, the embryo progresses through the midblastula

transistion (MBT), which is characterised by elongation of the cell cycle and zygotic transcription.

Extracts obtained from unfertilised oocytes recapitulate DNA replication in a way similar to the pre-MBT stages observed, where of course all the proteins neccessary for replication of DNA are to be found.

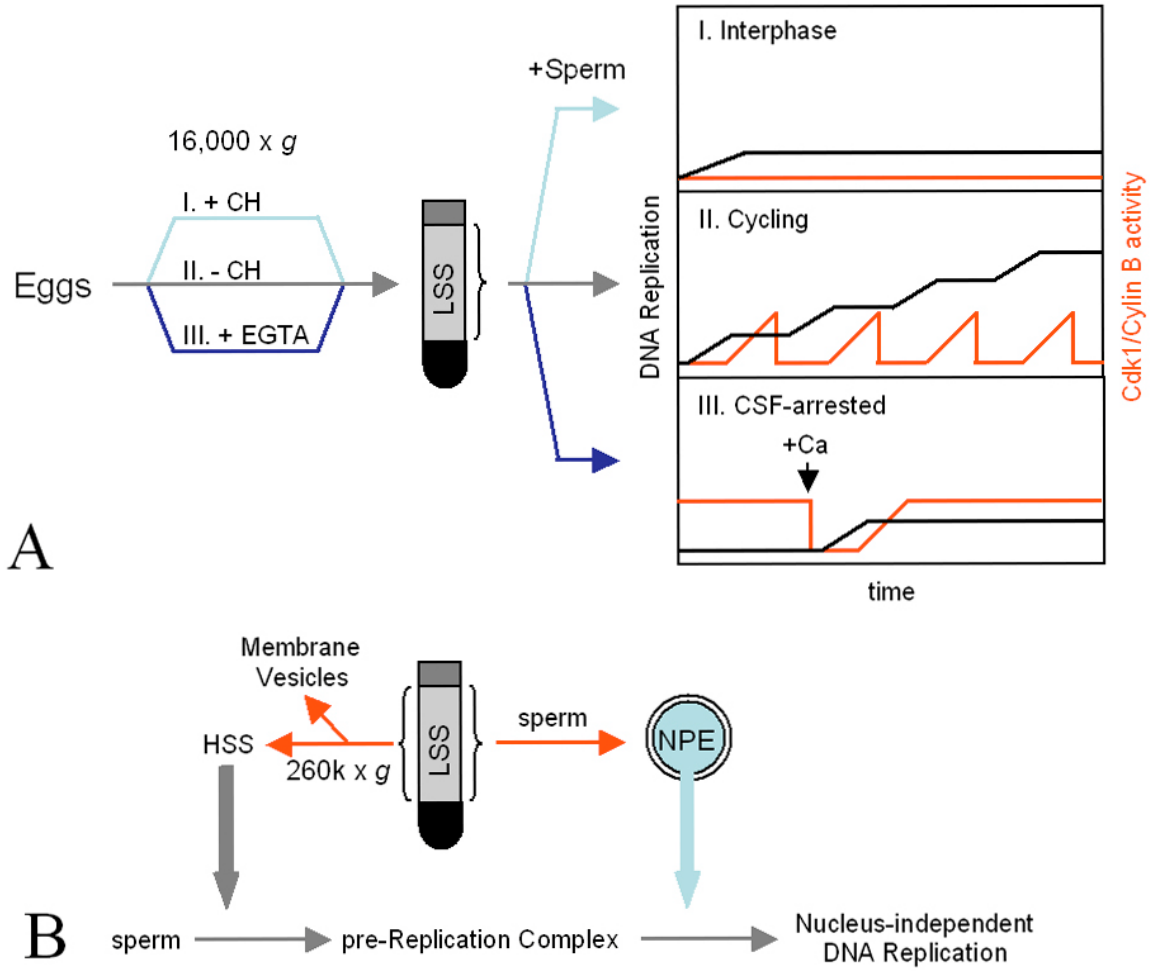


Figure 11. Schematic representation of the preparation of *Xenopus* egg extracts. A) Variants of crude cytoplasmic extracts (Low speed supernatant, LSS) that replicate upon sperm chromatin addition. Eggs are crushed in the presence of cycloheximide to impede Cyclin B resynthesis (I, Interphase extract), no additives (II, Cycling extract) or the calcium chelator EGTA (III, CSF-arrested). The graph indicates Cyclin B activity (red) and template replication (black.) B) Left arrow: High speed supernatant is harversted from crude cytoplasm (Low speed supernatant, LSS). This is used for pre-RC assembly. Right arrow: sperm chromatin is replicated in LSS to induce nuclei rich in protein content that is the fractionated into nucleoplasmid extract (NPE). This extract is added in excess to the reaction to initiate replication. Taken from [268].

Depending on the protocol used there are several ways to make extracts from these eggs [269, 270] (see Fig. 11). An LSS (low speed supernatant) extract is perhaps the simplest, obtained by crushing the unfertilised eggs in the presence of cycloheximide. Crushing leads to release of calcium, which mimicks sperm entry into the egg, degradation of Cyclin B and entry into interphase [271]. These extracts have been shown to replicate plasmid DNA to a certain extent [272, 273] but are mainly used for replication of demembranated sperm chromatin.

Replication of the chromatin involves formation of a transport competent nuclear envelope or "pseudo-nucleus" that accumulates high concentration of components required for replication. Traditionally, LSS can be further fractionated into a high speed supernatant (HSS) and a fraction of nuclear membrane vesicles, which allows separation of pre-RC assembly from replication initiation. Within the scope of this thesis, however, what is refered to as "S phase extract" is a variant of the HSS extract that does not require external nuclear membrane particles. As is the case for other *Xenopus* egg extracts, only a single round of semi-conservative replication is completed due to the presence of Geminin and degradation of Cdt1 [274]. Omision of cycloheximide during extract preparation is the protocol used for cycling extracts which can re-synthesize Cyclin B and can therefore re-enter alternating mitosis and S phase cycles up to 5 times. Another possibility is to add EGTA to the crushed eggs. The lack of calcium release means these CSF-arrested extracts are trapped in metaphase of meiosis II with high Cyclin B activity in a highly condensed state characteristic of meiosis.

Another form of extract is comprised by a combination of HSS extract and an enriched fraction obtained by harvesting replicating nuclei from LSS. This is known as NPE [275]. This system is particularly useful for the replication of small plasmid molecules (see Fig. 12)

A powerful way in which these extracts are employed is by depleting a protein of interest from the extract and measuring the effect on replication. This, combined with synchronicity of the reaction and the ease of adding chemicals or damaged templates that may interfere with extract activity emphasizes their value for biochemical studies on

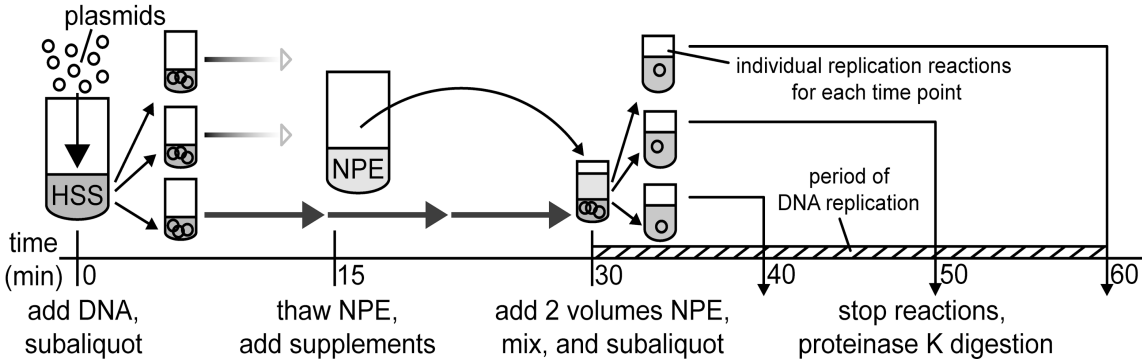


Figure 12. Schematic representation of DNA plasmid replication in NPE. The flowchart depicts a plasmid replication using NPE for 3 different time points. Plasmid is added to the HSS aliquot for pre-RC assembly. Only upon addition of 2 volumes of NPE to the reaction does replication begin. Taken from [276].

different aspects of DNA replication. The role of MCM7 and Cdc45 in origin unwinding during replication was studied this way, for example [277]. Similarly, recent studies have uncovered and supported replicative tasks for proteins such as the Tipin/Tim/And1 complex [278], Treslin, a novel TopBP1 interactor [279], and Rad51 [280-282].

This modus operandi extends to the role of DNA repair proteins in the context of replication, in particular proteins involved in crosslink- and double-strand break repair [186, 283] [284-286], as well as the coordination and activation of the DNA damage response in these extracts. Noteworthy with regards to this thesis is the fact the these extracts have been shown to be proficient in ATR dependent Chk1 activation in response to different types of damage [245, 287] [288-290]. Because of this, we postulated that it would be a valuable tool in studying MMR-dependent responses to alkylating damage in DNA.

4 Aims of this study

This introduction has elaborated on eukaryotic DNA replication and the varied DNA repair pathways that are key to maintaining genome stability by counteracting various sources of endogenous and exogenous DNA damage. Also described are the main signaling networks involved in halting and coordinating cell cycle progression in cases when the damage-induced stress proves to be too severe for a cell. Particular attention was paid to mismatch repair and its role in the DNA damage response to several cytotoxic agents as well as to the tool used in this study, the *Xenopus* egg extract system.

The aim of this PhD thesis was to gain insights into the mechanism of mismatch repair (MMR) dependent DNA damage signaling caused by genotoxic agents and chemotherapeutics such as S_N1-type methylating agents. Conflicting hypotheses as to the nature of the lesion recognition by the MMR machinery exist. The question as to whether it is directly involved in recruitment of the ATR pathway evidenced the need for a biochemical analysis of MMR protein dynamics during replication of undamaged and alkylation-damaged DNA. To date, both models addressing this, the direct signaling hypothesis and the futile cycling hypothesis, have reached a non-mutually exclusive *impasse*, so we aimed to clarify some open questions. The approach we took was to use the *Xenopus* egg extract (XEE) technique, permissive for the *in vitro* replication of DNA templates and hence recapitulates an *in vivo* replication scenario to a great extent. A limitation of this system was the lack of comercially available antibodies against *Xenopus* mismatch repair proteins so we first undertook the generation of a set of antibodies against several *Xenopus* MMR proteins. Namely, Msh2, Msh6, Mlh1 and Exo1. The second, and main aim of this thesis, was to analyze MMR activity on alkylation-generated O6-methylguanine (^{me}G) lesions both in sperm chromatin and lesion-specific plasmids during a single-round of synchronised DNA replication. This, with focus on possible parallel activation of the ATR checkpoint machinery in response to O6-methylguanine (^{me}G) recognition.

We also wanted to confirm the formation of MMR-associated gapped intermediates towards the end of replication and use them for a second round of replication. To our knowledge, ours is the only system that can monitor the replication of ^{me}G-containing

substrates and therefore also the associated formation of new ^{me}G mismatches. Collectively, these observations would finally pinpoint the importance of MMR intermediates in the G2 arrest during the second cell cycle following treatment with S_N1-type alkylators. Fully understanding the mode of action of these agents and how and why they bring about cell death should be a required platform for the continued use of these agents in chemotherapy and also the development of new cytotoxic drugs.

5 Results

5.1 Mismatch repair dependent processing of O6-methylguanine adducts in *Xenopus* egg extracts

Maite Olivera Harris, Mariela Artola Boran, Vincenzo Costanzo, Milica Enoiu and Josef Jiricny*

This manuscript describes the contribution of ^{me}G towards the toxicity of alkylating agents such as MNNG and MNU, which is associated with a MMR-dependent replication checkpoint. To study this phenomenon, we utilized extracts of *Xenopus laevis* eggs, a naturally synchronous *in vitro* replication system that not only allows replication of damaged chromatin, but also permits the study of the fate of ^{me}G-containing substrates during replication in the absence of other lesions. There is some dispute as to why ^{me}G adducts in DNA ultimately lead to cell death during a second cell cycle and not during the first. We found that replication of alkylated chromatin or of ^{me}G-containing substrates did not lead to recruitment of proteins in the ATR pathway and to activation of a ^{me}G-specific checkpoint. More importantly, we were able to detect single-stranded gaps in DNA caused by the presence of a single ^{me}G after replication completion. These would hinder a second round of replication in a way that the presence of the mismatches alone would not.

I contributed to this study by designing, performing and quantifying the research under the guidance and supervision of Josef Jiricny, as well as by drafting the manuscript.

Mismatch repair dependent processing of O6-methylguanine adducts in *Xenopus* egg extracts

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Keywords: Mismatch repair, S_N1-type alkylators, DNA damage signaling, DNA replication

Running title: The response to S_N1-type alkylators in XEE

Abstract: 100 words

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Abstract

Cytotoxicity by S_N1-type alkylators is largely ^{me}G dependent and catalyzed by MMR. Whether processing of the adducts during replication underlies the cytotoxicity remains unresolved. Here, we show that recruitment of the ATR checkpoint machinery to sites of alkylation damage does not coincide with that of MMR proteins in XEE. Furthermore, an accumulation of ^{me}G/T mispairs at the end of replication, a preferred MMR substrate, is not enough to trigger prolongued checkpoint activation. Also, ^{me}G-dependent gaps arise during replication and hinder re-replication in the extract. This suggests that the main cytotoxic trigger would be the attempt to replicate ^{me}G-containing gapped DNA.

Introduction

The mismatch repair system is assigned to eliminate mutations that might perturb genome stability [1]. As such, it recognizes mismatches and short insertion/deletion loops (IDLs) [2] formed during replication and directs its excision and resynthesis efforts to the nascent strand, in order to restore the correct coding information in our DNA. This task is mainly carried out by MutSα and MutLα (heterodimers of MSH2/MSH6 and MLH1/PMS2, respectively), EXO1, PCNA, RPA, RFC and polymerase δ [3] . From a clinical standpoint, it is also involved in checkpoint and apoptotic responses to chemotherapeutics such as S_N1-type methylating agents [4]. ^{me}G residues generated by these agents are normally detoxified by the suicide enzyme O6-methylguanine methyltransferase (MGMT) *via* direct removal of the methyl group from the guanine. When MGMT is absent, however, MMR-proficiency renders cells up to 100-fold more sensitive to S_N1-type methylators [5]. This methylation-biased phenomenon is characterized by MMR-dependent activation of the ATR and ATM checkpoint machineries, arrest and cell death in G2 of the second cell cycle [6, 7]. At the basis of this lies MMR recognition of ^{me}G/C and ^{me}G/T mispairs formed upon incorporation of a C or a T opposite ^{me}G by replicative polymerases. They are both bound by the MutSα heterodimer and it could be shown that removal of ^{me}G mispairs before entry into the second cell cycle is sufficient to disable the arrest [8, 9]. To date, two plausible explanations for how MMR machinery could address this damage exist. Since ^{me}G persists in the template strand, ongoing recognition and processing attempts by MMR behind the replication fork would create intermediates that, encountered during a second S phase, would prompt replication stalling. This is known as the futile cycling hypothesis [10, 11]. In accordance with this, a full complement of MutLα, which is downstream of mispair recognition by MutSα and which fine-tunes EXO1-catalyzed degradation of the error-containing strand, is needed for the methylation-induced arrest [12]. An alternate view, the direct signaling model, regards MutSα as a damage sensor and mediator of ATR activation [13], and suggests that mismatch recognition upon treatment with an S_N1-type alkylator is accountable for the observed toxicity. In support of this, interactions between MMR proteins and proteins of the ATR checkpoint machinery have been reported in co-immunoprecipitation studies and also *in vitro* using recombinant proteins [14, 15]. Nevertheless, it still remains unclear why repercussions of MMR recognition of

the ^{me}G-containing mismatches during the first S phase should unfold only during the second round of replication.

Recent reports from the Constanzo and Cimprich laboratories demonstrated the usefulness of the *Xenopus laevis* egg extract (XEE) in the study of replication-dependent DNA damage signaling [16, 17]. We therefore set out to use this system to study the response to S_N1-type alkylators during *in vitro* DNA replication. We report here that recruitment of MMR proteins to alkylation-damaged chromatin does not coincide with recruitment of the ATR checkpoint machinery. Furthermore, a closer examination of replication of methylated substrates revealed the presence of persistent single-stranded gaps opposite ^{me}G. Such gaps would cause the collapse of the replication forks during a subsequent S phase. Our data thus provide support for the futile processing hypothesis.

Results and discussion

MMR activity during replication in XEE was explored by generating a set of antibodies against the MMR proteins Msh2, Msh6, Mlh1 and Exo1 (Fig. S1A, B, C). Since MMR is believed to be closely associated with the replication fork [18], we first monitored replication-dependent recruitment of MMR proteins to undamaged sperm chromatin in S phase extracts (Fig. 1A). As shown in Fig. 1B, recruitment of Msh2, Msh6 and Exo1 was similar to that of the leading strand polymerase, pol ε. Interestingly, the residence time of Mlh1 in chromatin was substantially longer, which corresponds with reports that this protein has additional functions, particularly in recombination [19]. Since we were interested in studying damage response to S_N1-type alkylators in the extract, we then examined the effect of treating sperm chromatin with MNU, and monitoring both the recruitment of MMR proteins to the chromatin (Fig. 1A, C), as well as the replication products (Fig. 1D). MMR proteins could be seen to be recruited to chromatin in a ^{me}G-dependent fashion, as shown by increased amounts of these proteins in chromatin samples incubated with extracts pre-treated with Patrin-2, which inhibits Mgmt and thus ensures that ^{me}G persists in the DNA (Fig. 1C). As shown in Fig. 1D, MNU doses ranging from 0.5 to 4 mM did not visibly affect replication. Similar experiments with MNNG-treated chromatin (Fig. S2A, B, D), confirmed that, at doses which do not affect replication, MMR proteins are recruited to the chromatin together with replicative proteins such as pol ε and persist because of ^{me}G presence in the DNA.

Recently, MMR proteins were described as possible DNA damage sensors that can directly interact with the ATR checkpoint machinery upon ^{me}G recognition [13, 14]. These studies proposed a non-canonical activation of ATR and CHK1 in response to the lesion, which can occur independently of RPA or 9-1-1 complex recruitment. Since it would imply parallel recruitment of MMR and checkpoint machinery during replication, we used the naturally synchronous XEE as a platform for monitoring such recruitment during a single round of replication. To this end, sperm chromatin treated with increasing doses of MNU was replicated in extracts where Mgmt had been inhibited with Patrin-2. Treatment with MMS, which is known to elicit a checkpoint response in the extract, strongly recruits both MMR proteins as well as ATR machinery proteins. Contrary to this, there was no obvious recruitment of any ATR checkpoint proteins to the chromatin in response to MNU, even at doses that led to similar MMR recruitment as seen with MMS. This underscored even further that recognition of the ^{me}G throughout replication, as evidenced by the persistence of MMR proteins in the chromatin at later replication time points, does not necessarily trigger a checkpoint response. In accordance with this, no clear checkpoint activation (p-CHK1) could be detected at doses up to 4 mM MNU, i.e. at concentrations that do not affect replication (Fig. 2B). The treatment can trigger activation of CHK1, but only at high doses of MNU (80-500 mM) and in a manner that is independent of MMR (Fig. 2B, D).

One hypothesis explaining MMR-dependent cytotoxicity upon treatment with S_N1-type alkylators posits that formation of ^{me}G/T mismatches, arising through incorporation of a T opposite ^{me}G by the replicative polymerases, underlies the recruitment and activation of checkpoint responses. This agrees with reports that ^{me}G/T is a better MMR substrate than ^{me}G/C [20, 21], as also confirmed by us in *Xenopus* MMR assays which showed a MutSα-dependent MMR activity on the substrates (Fig. 3A, B, C). Of course, separating the effects of ^{me}G/C and ^{me}G/T in DNA during replication or even attempting to monitor generation of ^{me}G/T mismatches is not possible in cells. Fortunately, the XEE system offers the possibility of replicating plasmids containing single lesions of interest. We made use of this and switched to the HSS+NPE system for plasmid replication in XEE, where incubation of a substrate in two different extracts replicates it with high efficiency. We therefore decided to first replicate all MMR substrates (T/G, ^{me}G/C and ^{me}G/T) as well as a C/G control substrate in the extract. As can be seen in Fig. 3D, replication was complete already after 60 min and its efficiency was similar for all substrates and was

unaffected by the presence of the mismatches in the DNA. The presence of ^{me}G in the DNA substrates after replication was confirmed by dot blot with an antibody against ^{me}G.
Since our previous observations concerning the possible activation of CHK1 in response to ^{me}G was performed with MNU-treated sperm chromatin, we wanted to have a closer look at a possible checkpoint response during replication of a plasmid containing a single ^{me}G. Interestingly, we could observe transient ^{me}G-dependent checkpoint activation, but, as in the case of the MNU-treated sperm chromatin, this initial recognition was not tantamount to a prolonged checkpoint response (Fig. 4A). We cannot rule out that this transient activation was due to communication of MMR with the checkpoint machinery. In fact, recent work also showed that ^{me}G/T recognition can lead to activation of signaling *in vivo [22].* This, however, was not enough to sensitize cells to killing by the methylating agent and the downregulation of the effector protein involved had no effect on the MMR-dependent G2/M arrest, which would strongly argue in favor of a scenario where the ^{me}G/T mismatch needs to be processed to impart cytotoxicity.
Because there seemed to be no major involvement of the ATR checkpoint machinery in the response to ^{me}G, we wondered whether something structural in the DNA, independent of checkpoint signaling, could arise from replication of ^{me}G containing DNA. Although gaps present behind the replication fork in MNNG treated cells have been previously reported [9], a formal proof of ^{me}G lesions directly leading to gapped DNA was missing. We wondered whether the difference between the response during the first and second cell cycle in cells damaged with S_N1-type alkylators could be attributable to transient gaps formed by MMR futile cycling behind the replication fork that would be encountered during a second S phase. We performed a gap-filling assay post-replicatively (Fig. 4B). That is, we replicated the C/G and ^{me}G/C substrates without addition of a radioactive nucleotide to the reaction (a small control sample was removed for confirmation of replication), added a low dose of aphidicolin, a polymerase inhibitor, for a few minutes after replication was complete and then conducted a fill-in reaction with Klenow polymerase in the presence of [α-^{32}P]dCTP. The difference between the 2 substrates was clear: only the ^{me}G/C substrate contained gaps that could be filled-in. Furthermore, the majority of the radioactivity was found in the substrate fragment that contains the ^{me}G, indicative of post-replicative attempts by MMR to repair the lesion. This was substantiated by the detection of ^{me}G in the substrate after replication completion (Fig. 4C, Dot blot), confirming its status as an irreparable lesion. Finally, to confirm

whether the presence of these ^{me}G-dependent gaps could indeed impair replication if present in the template to be replicated, we conducted a re-replication reaction with sperm chromatin in the extract. This showed us that, indeed, a significant impairment of re-replication could be directly linked to the presence of ^{me}G-containing template in the extract (Patrin-2 treated). where MMR has more access to the lesion.

In conclusion, we were able to demonstrate that the presence of ^{me}G in DNA does not hinder replication, but that this modified base activates MMR. Because ^{me}G is in the template strand, it cannot be removed by the MMR reaction. This leads to futile attempts at repair/resynthesis, which leave gaps opposite the ^{me}G. These gaps do not signal to the checkpoint machinery, possibly due to being “masked” by RPA but they are sufficient to impair fork progression during a second round of replication.

Materials and methods

DNA substrates and *Xenopus laevis* MMR (xMMR) assay

The substrates were generated as described previously [23]. Briefly, the hetero- and homoduplexes were constructed by primer extension using the oligonucleotides listed below as primers and the single-stranded phagemid DNA as template. pRichi2850top*Sal*I (pRichi2850t *Sal*I) and pRichi2850top (pRichi2850t) that create 5' substrates were used as the ssDNA template. A single mutation abrogating an *Acl*I site and creating a *Sal*I site was used to generate the pRichi2850top *Sal*I phagemid. After primer extension and ligation, the desired supercoiled heteroduplex substrates were purified on CsCl gradients.

Mismatch-provoked excision assays were performed as previously described [24] in the presence of 100 ng DNA substrate and 26 µl HSS extract in a total volume of 30 µl in a buffer containing 15 mM Hepes-KOH pH 7.4, 15 mM potassium glutamate and 8.75 mM magnesium acetate, and 2 µCi of [α-^{32}P]dCTP. The extracts were incubated at 23°C for 45 min. The reaction was stopped by adding 70 µl STOP solution containing 1 mM EDTA, 3% SDS and 5 mg/ml proteinase K (Roche). The samples were incubated at 37°C ON, purified on Mini-Clean columns (Qiagen) and subjected to restriction digests in the presence of RNase (5prime). The digested DNA was cleaned up again and resolved on 1% agarose gels stained with GelRed.

Primers

All primers were obtained from Microsynth (Balgach, Switzerland). The sequences are indicated below. The *Sal*I restriction site (GTCGAC) is underlined. The mispaired residue is highlighted in bold.

C/G-*Sal*I primer:

5' CCAGACGTCTGT**C**GACGTTGGGAAGCTTGAG 3'

T/G-*Sal*I primer:

5' CCAGACGTCTGT**T**GACGTTGGGAAGCTTGAG 3'

^{me}G/C and ^{me}G/T *-SalI primer:

5' CCAGACGTCTGTC^{me}**G**ACGTTGGGAAGCTTGAG 3'

***** pRichi2850top *AclI* was used for this substrate to pair a T opposite the ^{me}G

***Xenopus laevis* egg extracts and chromatin isolation**

Lysolecithin-permeabilised *Xenopus laevis* sperm nuclei were prepared as described [25]. S phase extract capable of performing a single round of replication was prepared as previously described [26]. Briefly, eggs were dejelled, activated with calcium ionophore (Sigma), rinsed with S buffer (50 mM Hepes-KOH pH 7.5, 50 mM KCl, 2.5 mM $MgCl_2$ and 250 mM sucrose), transferred to 2 ml Eppendorf tubes and crushed by centrifugation for 12 min at 13 200 rpm. The cytoplasmic layer was removed and after addition of CytB (Sigma) cleared by centrifuging for 25 min at 70 000 rpm (TL55 swinging bucket rotor). Extract was supplemented with 250 μg/μl cycloheximide, 25 mM phosphocreatine and 10 μg/ml creatine phosphokinase before use.

To generate damaged chromatin, sperm chromatin was exposed to varying doses of MNU (Sigma), MNNG (Sigma) or 1% MMS (Sigma).

Egg cytosol (HSS) and NPE were prepared as described previously [27]. To make NPE, unfractionated egg extract (crude S phase extract) was supplemented with nocodazole (3.3 μg/ml), cycloheximide (50 μg/ml), DTT (1mM), aprotinin/leupeptin (10 μg/ml each) and cytochalasin B (5 μg/ml), and centrifuged at 10 000 rpm for 15 min in an HB-6 rotor at 4°C. The dark brown layer at the top of the tube was removed by aspiration and the extract was decanted into a fresh Falcon tube and supplemented with an ATP

regeneration system and demembranated sperm chromatin to a concentration of 4000 n/μl. The mixture was incubated at room temperature for 75-90 min. To collect nuclei, the reaction was centrifuged for 2 min at 10 000 rpm at 4°C in an HB-6. The clear ~4mm layer of nuclei was removed from the top of the tube and transferred to 5 x 20 mm ultracentrifuge tubes. They were then centrifuged for 30 min at 55 000 rpm in a Beckman TL199 ultracentrifuge using a TL55 swinging bucket rotor. After centrifugation, lipids at the top of the sample were aspirated and the clear nucleoplasm was harvested.

Isolation of nuclear and chromatin fractions

For isolation of nuclear fractions, egg extracts containing 4000 n/μl were incubated at 23°C for 120 min unless otherwise indicated, diluted in 500 μl EB buffer (100 mM KCl, 2.5 mM $MgCl_2$ and 50 mM Hepes-KOH pH 7.5), overlaid on a 30% sucrose/EB buffer cushion and centrifuged for 5 min. The pellets were resuspended and centrifuged again.

To isolate chromatin, sperm nuclei (4000 n/μl) were added to egg extract for the indicated times. For Western blotting, samples were diluted in ten volumes of EB buffer containing 0.25% Nonidet P-40 and centrifuged through a 30% sucrose layer at 10 000 rpm for 5 min at 4°C using a fixed angle rotor (HB-6), washed twice with 500 μl EB buffer and centrifuged at 10 000 rpm for 1 min. The pellet was resuspended in Laemmli loading buffer, the proteins were resolved by either 4-15%, 7.5% or 10% SDS-PAGE and analyzed by Western blotting with specific antibodies as indicated. Unless otherwise indicated, Fermentas prestained marker was used as a size reference.

DNA replication

Replication of sperm DNA (4000 n/μl), was measured by monitoring the incorporation of [α-^{32}P]dCTP for 120 min at 23°C as previously described [28]. Treatments are indicated in the figure legends. 5 μl aliquots were removed and combined with 15 μl of STOP mix (6 mM EDTA, 0.13% phosphoric acid, 10% Ficoll, 5% SDS, 0.2% Bromphenol blue and 80 mM Tris-HCl pH 8). Proteinase K (Roche) was then added to a final concentration of 2 μg/μl for 1 h at 37°C. Samples were then electrophoresed on a 0.8% agarose/TAE gel, after which the gel was dried and analyzed on a PhosphoImager with ImageQuant software (GE Healthcare)

For plasmid replication, plasmid DNA pRichi2850t was initially incubated in 6 μl HSS for 30 min at 23°C, followed by addition of a 2-fold volume of NPE. DNA replication was assayed by [α-^{32}P]dCMP (^{32}P) incorporation as described [27]. All replication reactions were carried out in the presence of Patrin-2 (100-150 μM), unless otherwise indicated. For substrate recovery, 40 μl STOP solution (5% SDS, 80 mM Tris-HCl pH 8 and 5 mg/ml RNase) was added for 30 min 37°C following by Proteinase K treatment (1 mg/ml) ON at 37°C. Samples were purified on Mini-Clean columns (Qiagen) and subjected to restriction digests in the presence of RNase (0.5 mg/ml, 5prime). The digested DNA was cleaned up again and resolved on 1% agarose gels stained with GelRed.

Production of recombinant proteins

Fragments of *Xenopus leavis* MMR proteins and full-length *Xenopus laevis* Mgmt were PCR-amplified from EST clones obtained from Imagenes using primers containing restriction sites for *Nco*I and *Xho*I. The PCR products were then cloned in a pET28B(+) vector (Novagen). 6xHis-tagged protein fragments (Msh2F1: 258-404 aa, Mlh1F1: 615-744aa, Msh6F1: 128-249aa, Exo1F1: 344-450aa) were expressed in BL21 cells (Invitrogen) and purified on Ni-NTA agarose columns (Qiagen) according to the manufacturer's protocol.

Antibodies

Rabbit *Xenopus laevis* Msh2, Msh6, Mlh1 and Exo1 were generated in a facility belonging to the Institute of Laboratory Animal Science (University of Zurich) against recombinant 6xHis-tagged protein fragments expressed in bacteria. On average, two rabbits per fragment were injected. Immunoglobulins specific for the *Xenopus laevis* MMR proteins were affinity-purified using Hitrap NHS-activated HP (GE healthcare) according to standard protocols. Only Msh6 and Exo1 sera worked for immunodepletions.

Rabbit polyclonal antibodies against *Xenopus laevis* TopBP1, Atr, Rad9, Rad1, Hus1, Rad17, Orc2 and Pol ε were a gift from Julian Blow (Dundee University, Dundee, UK), Howard Lindsay (Lancaster University, Lancaster, England, UK), Shou Waga (Osaka University, Osaka, Japan), William Dunphy (Stanford University, Stanford, CA) and Karlene Cimprich (Stanford University, Stanford, CA). Antibodies against human PCNA

(Santa Cruz), MCM7 (Sigma), CHK1 (Santa Cruz) and RPA32 subunit (Abcam), which also recognize the *Xenopus* proteins, were obtained commercially. The antibody that recognizes phospho-S345 of *Xenopus laevis* p-CHK1 was purchased from Cell Signaling Technology. Anti-^{me}G (EM2-3) antibody was a kind gift of Juergen Thomale (Duisburg-Essen University, Germany)

Western blot procedures were performed using ECL or ECL advanced (GE Healthcare). Antibodies for Western blots were used at 1:1000 dilutions, unless otherwise specified.

Dot blots

The dot blot procedure was described previously in Technologies for detection of DNA damage and mutations (G.P. Pfeifer, 1996 edition). Briefly, 200 ng of pRichi2850t *Sal*I substrates were heat-denatured at 100°C for 10 min. 2 M Ammonium acetate was added to a final concentration of 1 M and the samples were blotted onto pre-wetted nitrocellulose. The membrane was rinsed in 5 x SSC (0.75 M sodium chloride and 75 mM trisodium citrate) and blocked for 1 h in PBS/0.5% Casein/0.1% deoxycholate for 1 h at RT. Anti-^{me}G antibody (EM2-3) was used at 1:10 000 dilution for 1 h at RT in the same solution. Membranes were washed with PBS/160 mM NaCl/0.1% Triton X-100 3 x 5 min, incubated with a secondary anti-mouse antibody, washed further 3 times and developed.

Band shift assay

The oligonucleotide heteroduplexes were created by annealing 5' labelled oligonucleotides (see **^{me}G/C and ^{me}G/T *-SalI primer** for oligonucleotide sequences) with complementary oligonucleotides containing a C or a T opposite the ^{me}G. Where indicated, annealed substrates were treated with Mgmt prior to band shift. The binding reaction mixtures (50mM Hepes pH 7.5, 0.5mM EDTA, 10% glycerol, 0.5mM DTT, 0.5mg/ml BSA and 20 ng polydI-C) contained 40 fmol oligonucleotide duplex ^{me}G/C or ^{me}G/T and human MSH2/MSH6 (250 ng). Protein-bound substrates were separated from free probes by electrophoresis on a 6% native polyacrylamide gel eluted with TAE.

***Xenopus laevis* MSH6 immunodepletion**

To prepare Msh6 depleted extracts, 100 μl extract were incubated 3 times for 30 min with 33 μl protein A-Sepharose beads 4B fast flow (Sigma) coupled to 100 μl of anti-Msh6

serum. Mock depletion was performed using beads coupled to pre-immune serum. The coupling to the beads was performed without diluting the serum ON at 4°C. After the coupling reaction, the beads were washed extensively with EB buffer. The extract was reconstituted with 1000 ng hMutSα where indicated.

Figure legends

Figure 1. Recruitment of *Xenopus* MMR proteins to sites of MNU-induced alkylation damage. A) Schematic representation of the chromatin association assay. B) Western blot showing a time course of recruitment of the indicated replication and MMR proteins to chromatin. C) Recruitment of the indicated proteins to chromatin of undamaged or MNU-treated (1.5 mM) sperm nuclei (4000 n/μl), incubated with the extracts for 120 min in the absence or presence of the Mgmt inhibitor Patrin-2 (150 μM). and then subjected to Western blotting. D) Efficiency of replication of MNU-treated sperm chromatin in S phase extract. Chromatin replication was monitored by supplementing the extracts with [α-^{32}P]dCTP (^{32}P) and incubation for 120 min with the MNU-treated nuclei (4000 n/μl). Left panel: Autoradiograph of a 0.8% agarose gel. Right panel: Column graph of data from three independent experiments, with error bars representing standard deviation from the mean.

Figure 2. Role of the ATR checkpoint machinery in replication of alkylation-damaged chromatin bound *Xenopus* MMR proteins. A) Western blot showing binding of mismatch repair proteins Msh2 and Mlh1 as well as Atr, Atrip, TopBP1, Rad17 and Hus1 to chromatin in response to a range of MNU-induced damage. MNU-treated sperm chromatin (4000 n/μl) was replicated in S phase extract and samples were removed 120 min post nuclei addition. Orc2 blotting was used as a loading control. Chromatin treated with MMS (1%) was replicated as a positive control for Atr activation. B) Phosphorylation of CHK1 (S345) in S phase extract in response to MNU. Nuclei were isolated at 120 min and probed for p-CHK1 (S345) and CHK1. C) MMR-independent phosphorylation of CHK1 (S345) in S phase extract in response to MNU. Nuclei were isolated at 120 min and probed for p-CHK1 (S345) and CHK1.

Figure 3. MMR activity on pRichie2850t substrates and their replication in XEE. A) Left: Schematic representation of a pRichi2850t substrate digested with *Hin*dIII/*Dra*I (H/D). All substrates contain a *Hin*dIII restriction site (AAGCTT, green) in close proximity

to the mismatch. B) xMMR assay with C/G, T/G, ^{me}G/C and ^{me}G/T pRichi2850t substrates. Substrates were incubated in HSS extract (See Methods) for 30 min at 22°C prior to *Hin*dIII/*Dra*I digestion. Top: Digested samples analyzed on a 1% agarose gel and stained with GelRed. Restriction fragments are indicated by **a*+b*** (2484bp), **a*** (1336bp), **b*** (1148) and **c*** (694bp). Bottom: Autoradiograph (^{32}P) of the agarose gel. Right: Data from three independent MMR assays are plotted, with error bars representing standard deviation from the mean. C) xMMR assay in HSS extract. Mock- and Msh6-depleted extract were incubated with ^{me}G/C pRichi2850t *Sal*I substrate (see Materials and methods) and loaded on a 1% agarose gel after *Sal*I/*Dra*I digestion. Restoration of MMR activity in Msh6-depleted extract was shown by the addition of recombinant human MutSα. Top: Digested samples analyzed on a 1% agarose gel and stained with GelRed. Restriction fragments are indicated by **a*+b*** (2484bp), **a*** (1336bp), **b*** (1148) and **c*** (694bp). Bottom: Autoradiograph (^{32}P) of the agarose gel. Right: Western blot showing depletion of Msh6 in the extract. D) Kinetic analysis of the substrate replication in HSS+NPE. The plasmids were incubated in HSS for 30 min for pre-RC formation, followed by addition of 2 vol. of NPE for initiation of replication (see Supplemental Information for details). Samples were taken from the reaction at the indicated time points, analyzed on a 0.8% agarose gel and autoradiographed (^{32}P). Lower panel: Dot blot against ^{me}G with the respective replicated substrates.

Figure 4. Replication of ^{me}G adducts in *XEE* leads to gapped-replication products that escape checkpoint monitoring and affect further replication. A) Kinetic analysis of checkpoint activation (p-CHK1) during substrate replication. Indicated substrates (C/G, ^{me}G/C and MMS-treated C/G) were replicated in HSS+NPE, samples were removed at the indicated times, resolved on a 7.5% SDS-PAGE and probed for p-CHK1 (S345) and CHK1. Time point 0 indicates addition of NPE to the HSS+DNA mix. B) Schematic representation ofa pRichi2850t substrate digestion with *Drd*I. C) Post-replivative fill-in of a control substrate (C/G) and a ^{me}G-containing substrate. Substrates were replicated without addition of radioactive nucleotide (control sample was spiked with [α-^{32}P]dCTP). Aphidicolin (7.5 μM) was added for 5 min after replication was complete, the substrate was isolated and a fill-in reaction with Klenow polymerase was performed in the presence of [α-^{32}P]dCTP. Substrates were then digested with *Drd*I (Dr) and the samples were run on a 1% GelRed-stained agarose gel. Bottom: autoradiograph (^{32}P) and dot blot

against ^{me}G of the replicated substrates. D) Effect of ^{me}G presence on re-replication of undamaged and MNU-damaged sperm chromatin as indicated by autoradiograph (^{32}P). Damaged or undamaged samples were replicated in 1st extract (DMSO-treated or Patrin-2 treated), isolated and introduced into 2nd extract (Patrin-2 treated). One sample was treated with ExoIII after 1st extract replication as a positive control for impaired re-replication.

Fig. S1. Generation of antibodies specific for *Xenopus laevis* MMR proteins. A) BLAST search showing conservation between *Homo sapiens* and *Xenopus laevis* MMR proteins of interest. B) Coomasie-stained 6xHis-tagged xMMR protein fragments resolved on a 15% SDS-PAGE. Each lane was loaded with 1 µl of purified fragment F1 (Msh2F1: 1.5 µg/µl, Msh6F1: 1.5 µg/µl, Mlh1F1: 3 µg/µl, Exo1F1: 1.2 µg/µl). M, size marker in kDa. C) Western blot of *Xenopus laevis* egg extract (NPE, 1µl) with the affinity-purified xMMR antibodies using the following dilutions (Msh2: 1:5000, Msh6: 1:5000, Mlh1: 1:500, Exo1: 1:1000).

Fig. S2. Recruitment of *Xenopus* MMR proteins to sites of MNNG-induced alkylation damage in S phase extracts. A) Kinetic analysis of the replication of undamaged and MNNG-damaged sperm chromatin. Binding of Msh2 and Pol-ε to the chromatin was monitored by western blotting. B) ^{me}G-dependent recruitment of xMMR proteins to chromatin. Binding of mismatch repair proteins Msh2, Msh6 and Mlh1 to undamaged or MNNG-damaged chromatin in the absence or presence of the Mgmt inhibitor Patrin-2 samples was assayed at 120 min. E: extract. C) Monitoring checkpoint activation in XEE in response to MNNG (1.5 mM). Undamaged or MNNG-damaged sperm chromatin was replicated in S phase extract in the absence or presence of Patrin-2. Nuclei were isolated from this experiment at 120 min and probed for p-CHK1 (S345) and CHK1.

Figure S3. Purification of recombinant *Xenopus* Mgmt and quality control of ^{me}G-containing substrates in XEE. A) Coomassie staining showing the purification of full-length 6xHis-tagged Mgmt (25kDa). 15 µl of each fraction were resolved on a 15% SDS-PAGE. C, Whole-cell lysate from IPTG-induced BL21 cells carrying the xMMR constructs; FT, flow-through fraction of the nickel chelated affinity chromatography; W, wash fraction; Mgmt, fractions obtained after eluting with imidazole. B) Band shift assay with hMutSα for ^{me}G/C and ^{me}G/T substrates used in replication and repair assays.

Presence of a ^{me}G/C or ^{me}G/T mismatch was confirmed by Mgmt treatment prior to band shift reaction. C) Overview of pRichi2850t substrates depicting the position of the respective mismatches (T/G, ^{me}G/C and ^{me}G/T) with respect to the restriction sites used in this study (GTCGAC, *Sal*I, TTCGAA, *Hin*dIII). D) Schematic representation of a pRichi2850t substrate digested with *Sal*I/*Dra*I (S/D). E) *Sal*I/*Dra*I cleavage of pRichi2850t substrates C/G, T/G, ^{me}G/C and ^{me}G/T. Presence of a ^{me}G/C or ^{me}G/T mismatch was confirmed by Mgmt treatment prior to digestion. * indicates unspecific cleavage of ^{me}G/C substrate by *Sal*I which can be seen in a higher exposure of the GelRed-stained agarose gel.

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Olivera-Harris et al., Fig. 1

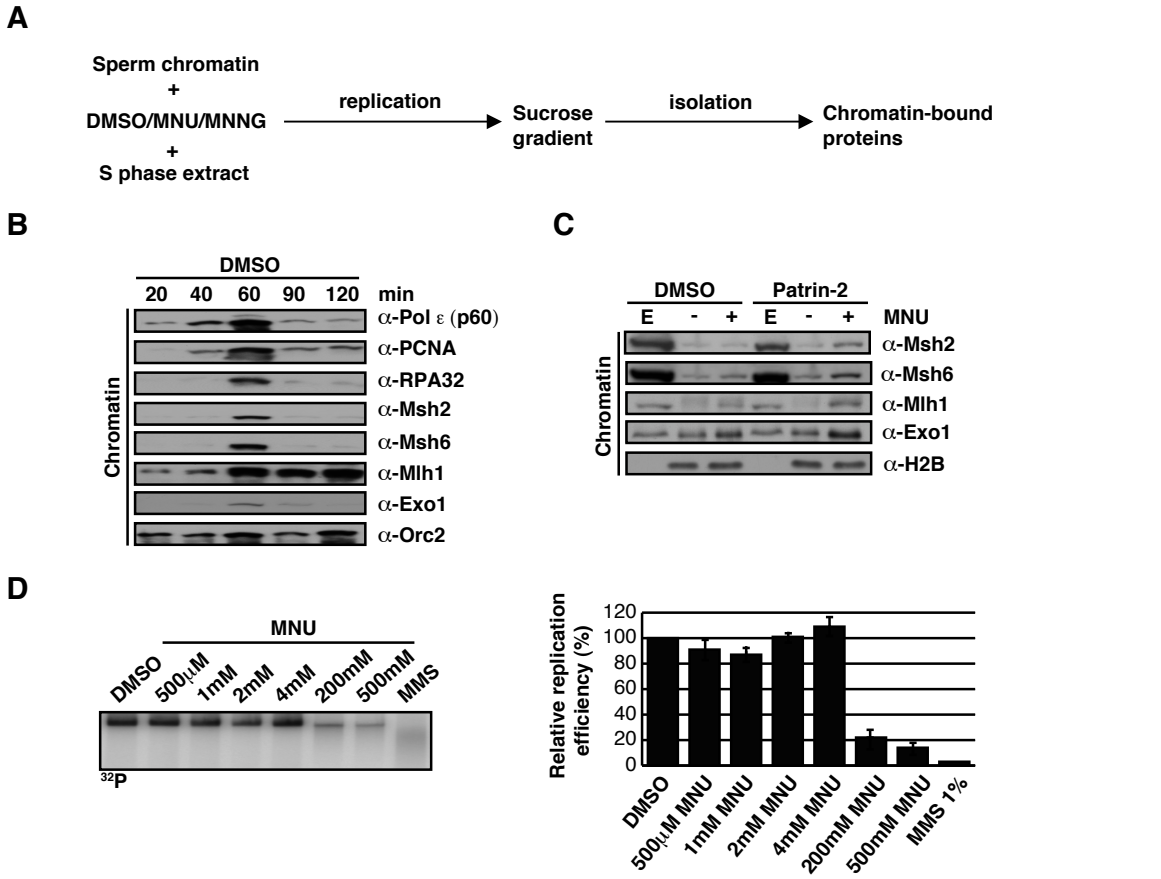


Figure 1. Recruitment of *Xenopus* MMR proteins to sites of MNU-induced alkylation damage. A) Schematic representation of the chromatin association assay. B) Western blot showing a time course of recruitment of the indicated replication and MMR proteins to chromatin. C) Recruitment of the indicated proteins to chromatin of undamaged or MNU-treated (1.5 mM) sperm nuclei (4000 n/μl), incubated with the extracts for 120 min in the absence or presence of the Mgmt inhibitor Patrin-2 (150 μM). and then subjected to Western blotting. D) Efficiency of replication of MNU-treated sperm chromatin in S phase extract. Chromatin replication was monitored by supplementing the extracts with [α-^{32}P]dCTP (^{32}P) and incubation for 120 min with the MNU-treated nuclei (4000 n/μl). Left panel: Autoradiograph of a 0.8% agarose gel. Right panel: Column graph of data from three independent experiments, with error bars representing standard deviation from the mean.

Olivera-Harris et al., Fig. 2

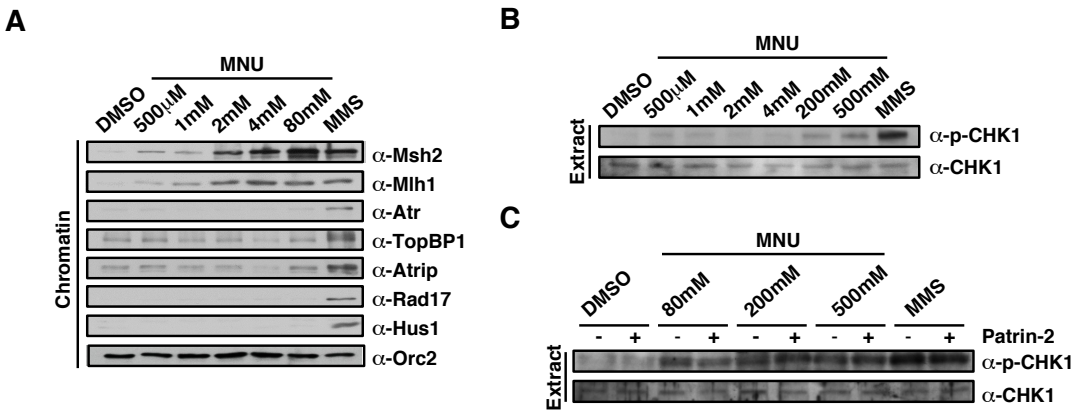


Figure 2. Role of the ATR checkpoint machinery in replication of alkylation-damaged chromatin bound *Xenopus* MMR proteins. A) Western blot showing binding of mismatch repair proteins Msh2 and Mlh1 as well as Atr, Atrip, TopBP1, Rad17 and Hus1 to chromatin in response to a range of MNU-induced damage. MNU-treated sperm chromatin (4000 n/μl) was replicated in S phase extract and samples were removed 120 min post nuclei addition. Orc2 blotting was used as a loading control. Chromatin treated with MMS (1%) was replicated as a positive control for Atr activation. B) Phosphorylation of CHK1 (S345) in S phase extract in response to MNU. Nuclei were isolated at 120 min and probed for p-CHK1 (S345) and CHK1. C) MMR-independent phosphorylation of CHK1 (S345) in S phase extract in response to MNU. Nuclei were isolated at 120 min and probed for p-CHK1 (S345) and CHK1.

Olivera-Harris et al., Fig. 3

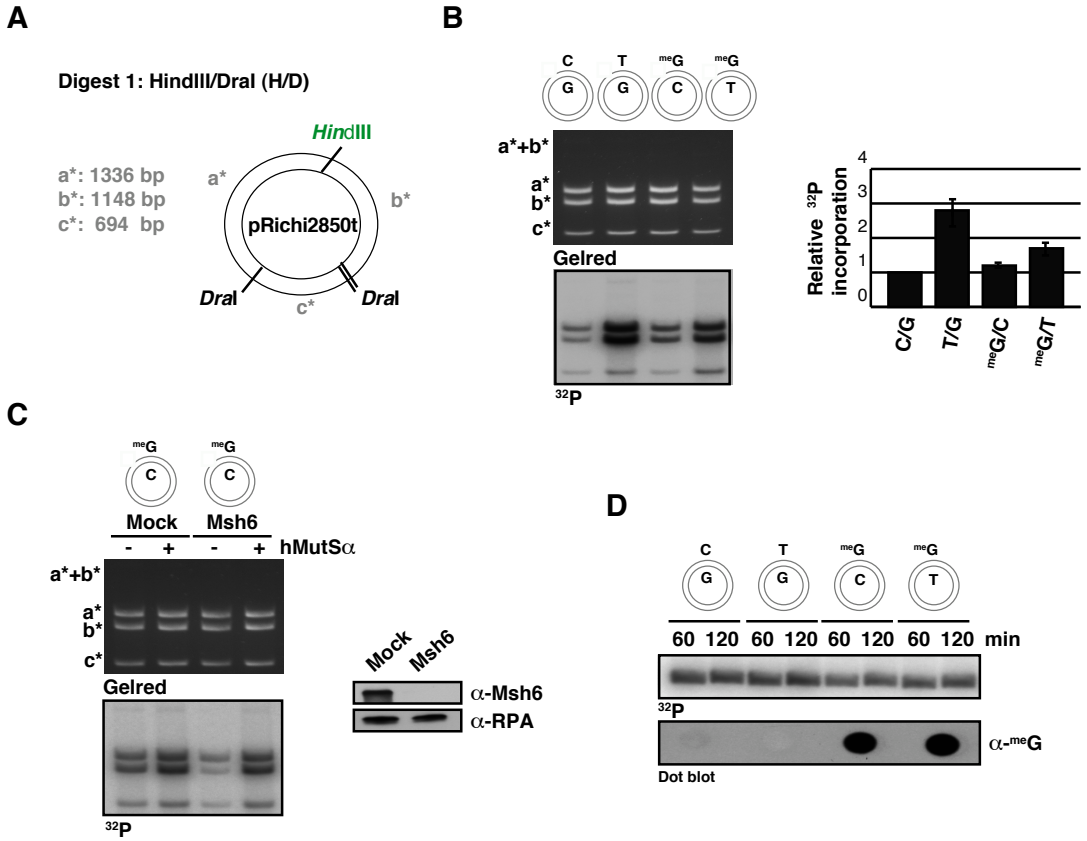


Figure 3. MMR activity on pRichie2850t substrates and their replication in XEE. A) Left: Schematic representation of a pRichi2850t substrate digested with *Hin*dIII/*Dra*I (H/D). All substrates contain a *Hin*dIII restriction site (AAGCTT, green) in close proximity to the mismatch. B) xMMR assay with C/G, T/G, ^{me}G/C and ^{me}G/T pRichi2850t substrates. Substrates were incubated in HSS extract (See Methods) for 30 min at 22°C prior to *Hin*dIII/*Dra*I digestion. Top: Digested samples analyzed on a 1% agarose gel and stained with GelRed. Restriction fragments are indicated by **a*+b*** (2484bp), **a*** (1336bp), **b*** (1148) and **c*** (694bp). Bottom: Autoradiograph (^{32}P) of the agarose gel. Right: Data from three independent MMR assays are plotted, with error bars representing standard deviation from the mean. C) xMMR assay in HSS extract. Mock- and Msh6-depleted extract were incubated with ^{me}G/C pRichi2850t *Sal*I substrate (see Materials and methods) and loaded on a 1% agarose gel after *Sal*I/*Dra*I digestion. Restoration of MMR activity in Msh6-depleted extract was shown by the addition of recombinant human MutSα. Top: Digested samples analyzed on a 1% agarose gel and stained with GelRed. Restriction fragments are indicated by **a*+b*** (2484bp), **a*** (1336bp), **b*** (1148) and **c*** (694bp). Bottom: Autoradiograph (^{32}P) of the agarose gel. Right: Western blot showing depletion of Msh6 in the extract. D) Kinetic analysis of the substrate replication in HSS+NPE. The plasmids were incubated in HSS for 30 min for pre-RC formation, followed by addition of 2 vol. of NPE for initiation of replication (see Supplemental Information for details). Samples were taken from the reaction at the indicated time points, analyzed on a 0.8% agarose gel and autoradiographed (^{32}P). Lower panel: Dot blot against ^{me}G with the respective replicated substrates.

Olivera-Harris et al., Fig. 4

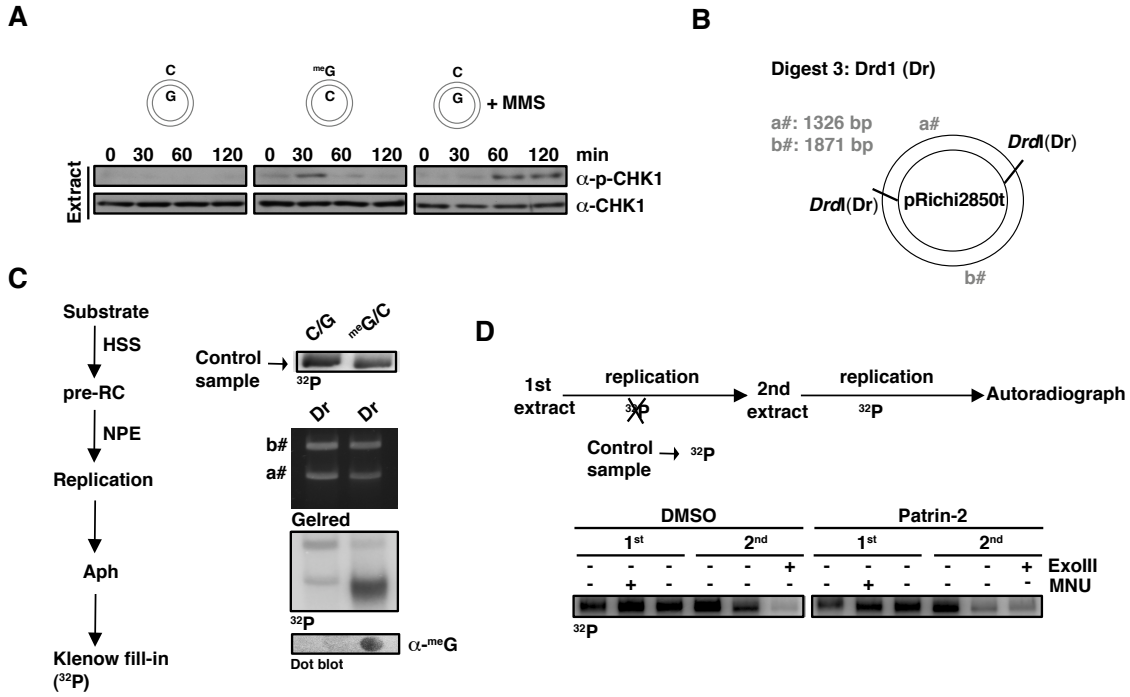


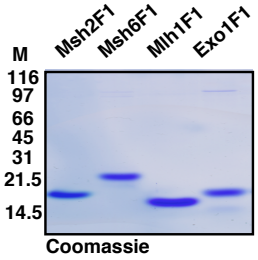
Figure 4. Replication of ^{me}G adducts in *XEE* leads to gapped-replication products that escape checkpoint monitoring and affect further replication. A) Kinetic analysis of checkpoint activation (p-CHK1) during substrate replication. Indicated substrates (C/G, ^{me}G/C and MMS-treated C/G) were replicated in HSS+NPE, samples were removed at the indicated times, resolved on a 7.5% SDS-PAGE and probed for p-CHK1 (S345) and CHK1. Time point 0 indicates addition of NPE to the HSS+DNA mix. B) Schematic representation ofa pRichi2850t substrate digestion with *Drd*I. C) Post-replivative fill-in of a control substrate (C/G) and a ^{me}G-containing substrate. Substrates were replicated without addition of radioactive nucleotide (control sample was spiked with [α-^{32}P]dCTP). Aphidicolin (7.5 μM) was added for 5 min after replication was complete, the substrate was isolated and a fill-in reaction with Klenow polymerase was performed in the presence of [α-^{32}P]dCTP. Substrates were then digested with *Drd*I (Dr) and the samples were run on a 1% GelRed-stained agarose gel. Bottom: autoradiograph (^{32}P) and dot blot against ^{me}G of the replicated substrates. D) Effect of ^{me}G presence on re-replication of undamaged and MNU-damaged sperm chromatin as indicated by autoradiograph (^{32}P). Damaged or undamaged samples were replicated in 1st extract (DMSO-treated or Patrin-2 treated), isolated and introduced into 2nd extract (Patrin-2 treated). One sample was treated with ExoIII after 1st extract replication as a positive control for impaired re-replication.

Olivera-Harris et al., Fig. S1

A

Xenopus MMR protein	Identity to human (%)
Msh2	79
Msh6	66
Mlh1	86
Exo1	52

B



C

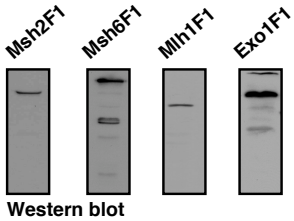


Fig. S1. Generation of antibodies specific for *Xenopus laevis* MMR proteins. A) BLAST search showing conservation between *Homo sapiens* and *Xenopus laevis* MMR proteins of interest. B) Coomasie-stained 6xHis-tagged xMMR protein fragments resolved on a 15% SDS-PAGE. Each lane was loaded with 1 µl of purified fragment F1 (Msh2F1: 1.5 µg/µl, Msh6F1: 1.5 µg/µl, Mlh1F1: 3 µg/µl, Exo1F1: 1.2 µg/µl). M, size marker in kDa. C) Western blot of *Xenopus laevis* egg extract (NPE, 1µl) with the affinity-purified xMMR antibodies using the following dilutions (Msh2: 1:5000, Msh6: 1:5000, Mlh1: 1:500, Exo1: 1:1000).

Olivera-Harris et al., Fig. S2

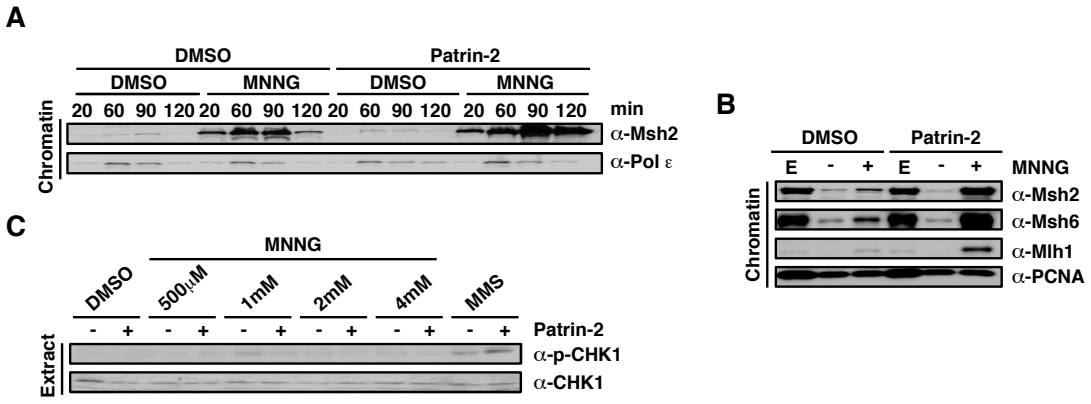


Fig. S2. Recruitment of *Xenopus* MMR proteins to sites of MNNG-induced alkylation damage in S phase extracts. A) Kinetic analysis of the replication of undamaged and MNNG-damaged sperm chromatin. Binding of Msh2 and Pol-ε to the chromatin was monitored by western blotting. B) ^{me}G-dependent recruitment of xMMR proteins to chromatin. Binding of mismatch repair proteins Msh2, Msh6 and Mlh1 to undamaged or MNNG-damaged chromatin in the absence or presence of the Mgmt inhibitor Patrin-2 samples was assayed at 120 min. E: extract. C) Monitoring checkpoint activation in XEE in response to MNNG (1.5 mM). Undamaged or MNNG-damaged sperm chromatin was replicated in S phase extract in the absence or presence of Patrin-2. Nuclei were isolated from this experiment at 120 min and probed for p-CHK1 (S345) and CHK1.

Olivera-Harris et al., Fig. S3

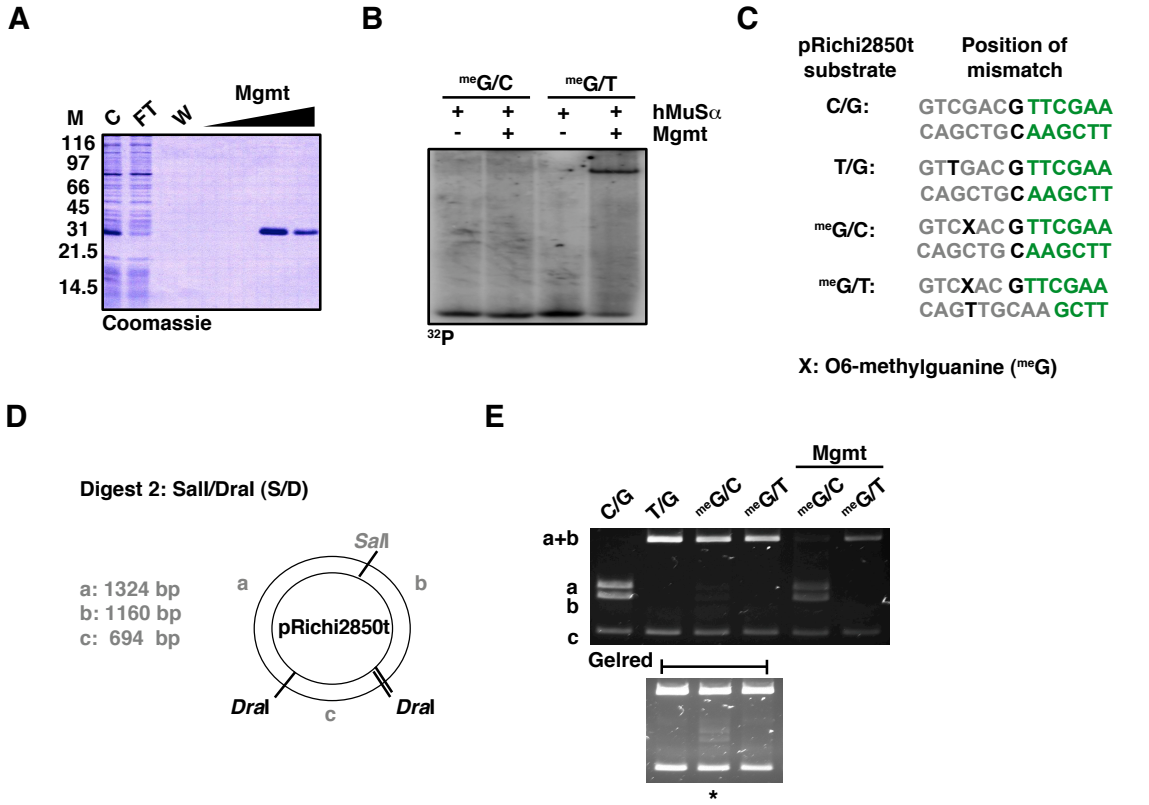


Figure S3. Purification of recombinant *Xenopus* Mgmt and quality control of ^{me}G-containing substrates in XEE. A) Coomassie staining showing the purification of full-length 6xHis-tagged Mgmt (25kDa). 15 µl of each fraction were resolved on a 15% SDS-PAGE. C, Whole-cell lysate from IPTG-induced BL21 cells carrying the xMMR constructs; FT, flow-through fraction of the nickel chelated affinity chromatography; W, wash fraction; Mgmt, fractions obtained after eluting with imidazole. B) Band shift assay with hMutSα for ^{me}G/C and ^{me}G/T substrates used in replication and repair assays. Presence of a ^{me}G/C or ^{me}G/T mismatch was confirmed by Mgmt treatment prior to band shift reaction. C) Overview of pRichi2850t substrates depicting the position of the respective mismatches (T/G, ^{me}G/C and ^{me}G/T) with respect to the restriction sites used in this study (GTCGAC, *Sal*I, TTCGAA, *Hin*dIII). D) Schematic representation of a pRichi2850t substrate digested with *Sal*I/*Dra*I (S/D). E) *Sal*I/*Dra*I cleavage of pRichi2850t substrates C/G, T/G, ^{me}G/C and ^{me}G/T. Presence of a ^{me}G/C or ^{me}G/T mismatch was confirmed by Mgmt treatment prior to digestion. * indicates unspecific cleavage of ^{me}G/C substrate by *Sal*I which can be seen in a higher exposure of the GelRed-stained agarose gel.

5.2 Tools generated to facilitate the study of mismatch repair in *Xenopus laevis* egg extracts

At the start of this PhD thesis, the main concern was establishing the *Xenopus laevis* facility in our institute. We had some problems with the maintenance of the frogs and this lead to difficulties with egg production. This, however, was overcome in due time with a stepwise reorganization of several factors crucial to the frogs well-being, e.g food and light source. Despite this unfortunate delay, we proceeded with the generation of antibodies specific for *Xenopus* MMR proteins. This step is a crucial and often overlooked requirement in working with Xenopus egg extracts (XEE). Many proteins of interest are expressed in the extract, but due to variations in homology between human and *Xenopus* proteins, commercially-available antibodies often do not recognize the *Xenopus* orthologs. Once this has been verified, as in our case where several antibodies failed to recognize the *Xenopus* proteins (data not shown), it is neccessary to generate your own set of antibodies.

In order to conduct a speedy and effective expression and purification of the proteins, small fragments of *Xenopus* MMR genes were first cloned into the pET28b(+) expression vector. Available cDNAs (partial Msh2 and full-length Msh6, Mlh1 and Exo1; Msh3 is unfortunately not anotated) had been previously purchased from Imagenes. This vector was chosen because of the possibility of tagging the fragments with a 6xHis-tag, which is known to be poorly immunogenic. The cloned *Xenopus* MMR fragments were then expressed in *E. coli* and, depending on their expression and solubility, a large scale expression was conducted, either under non-denaturing (Fig. 13A, B and D) or denaturing conditions (Fig. 13C). In the end, one fragment (F1) from each *Xenopus* protein (Msh2, Msh6, Mlh1 and Exo1) was selected for rabbit immunisation. Sufficient amounts of each antigen were obtained for rabbit immunisations, since less the 1 mg total protein is neccesary for the procedure and the immunisation was carried out in a nearby facility on the University of Zurich Campus.

Antiserum from an immunised animal can be used for certain applications, for immunodepletion in the XEE for example, which we could indeed use for succesful immunodepletion of Msh6 and Exo1 from the extract (Fig. 14A, B). For more specific detection methods, however, isolation of the antibody is useful. We therefore conducted antibody purifications for each MMR protein (Fig. 15A, B, C and D) with the final bleeds

of the rabbit sera. We used purified antigens as baits to capture antigen-specific immunoglobulins from the serum. This was done to avoid the drawback of Protein G/A-

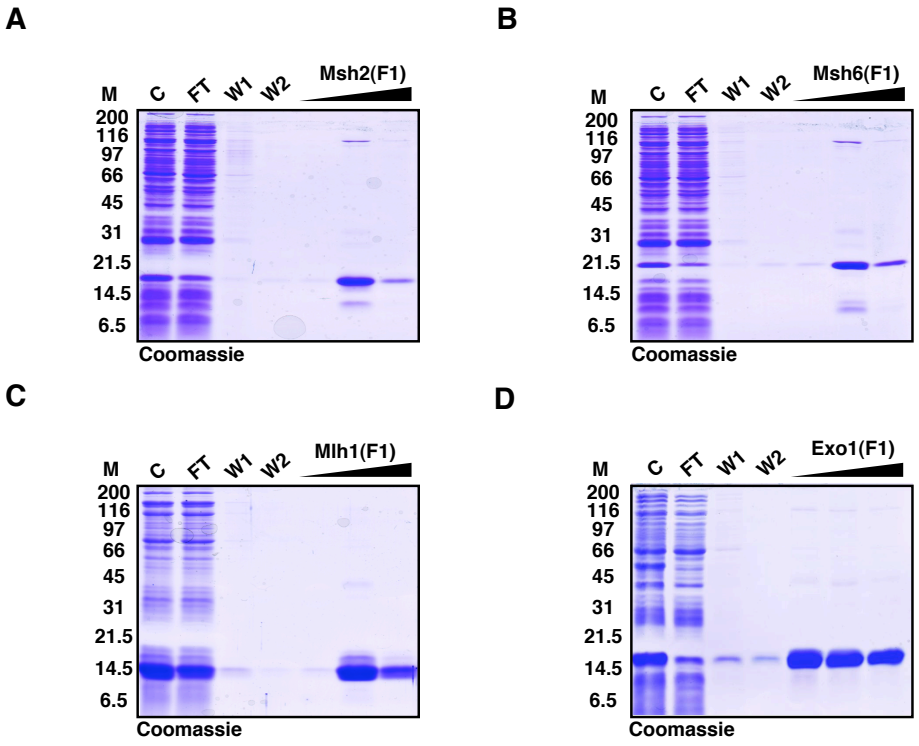


Fig 13. Purification of *Xenopus* MMR protein fragments expressed in *E. coli* (BL21). Coomasie staining showing samples obtained during the Ni-NTA affinity purification of 6xHis-tagged protein fragments resolved on a 15% SDS-PAGE. C, whole-cell lysate from IPTG-induced BL21 cells carrying the *Xenopus* MMR construct; FT, flow-through fraction of the nickel chelated affinity chromatography; W1/W2, washes; black triangle, elution fragments (Imidazole) obtained that were pooled and dialysed against 20 mM MOPS/100 mM NaCl prior to rabbit immunisation. M, size marker in kDa (Biorad broad range protein marker). A) Purification of Msh2, fragment 1 (258aa-404aa). B) Purification of Msh6, fragment 1 (128aa-249aa). C) Purification of Mlh1, fragment 1 (62aa-172aa). Denaturing conditions, see material and methods for details. D) Purification of Exo1, fragment 1 (344aa-450aa).

based purification methods, were the final preparation contains host IgG as well as the specific antibodies.

Once these purifications were standardised, a similar expression and purification protocol was followed to purify recombinant Geminin and p27 (Fig. 16A, B), two proteins which inhibit replication in the *Xenopus* extract. Geminin acts by preventing loading of the MCM complex onto chromatin and p27 is a CDK inhibitor. Activity of the proteins was

confirmed in an *in vitro* replication assay in the extract (Fig. 16C) and they were then used in preliminary experiments. These results confirmed that MMR proteins could be recruited to chromatin outside of replication (Fig. 17A) and that, in general, ubiquitination of PCNA could be detected in the extract during replication, but more persistantly upon induction of exogenous damage (Fig. 17B, C).

Preliminary experiments conducted in the XEE show that replication of not only ^{me}G-containing substrates but also of a T/G mismatch can lead to transient checkpoint activation (Fig. 18) and would be interesting to investigate further with regard to what checkpoint machinery proteins are recruited to the ^{me}G together with MMR. This could be achieved by replicating biotinylated substrates in the extract and attempting to chip proteins specifically bound to the ^{me}G lesion with the use of streptavidin beads. Replication of the biotinylates substrates in HSS+NPE extract does not affect replication to a great extent when compared to a non-biotinylated substrate (Fig. 19).

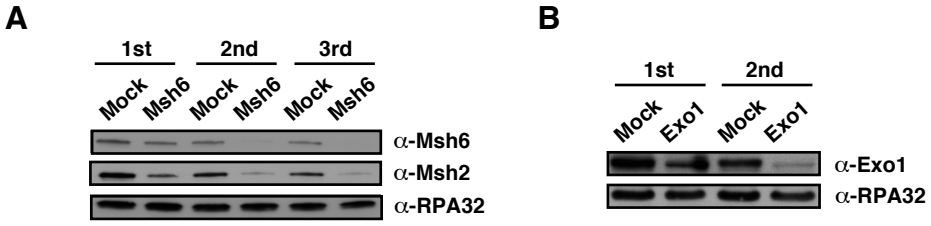


Fig. 14. Immunodepletion of Msh6 and Exo1 from S phase extracts. A) Mock-depleted (Mock) and Msh6-depleted (Msh6) extract after the first (1st), second (2nd) and third (3rd) round of immunodepletion using Msh6 serum prebound to protein A Sepharose beads. B) Mock-depleted (Mock) and Exo1-depleted (Exo1) extract after the first (1st) and second (2nd) round of immunodepletion using Exo1 serum prebound to protein A-Sepharose beads. Samples were blotted with the indicated antibodies.

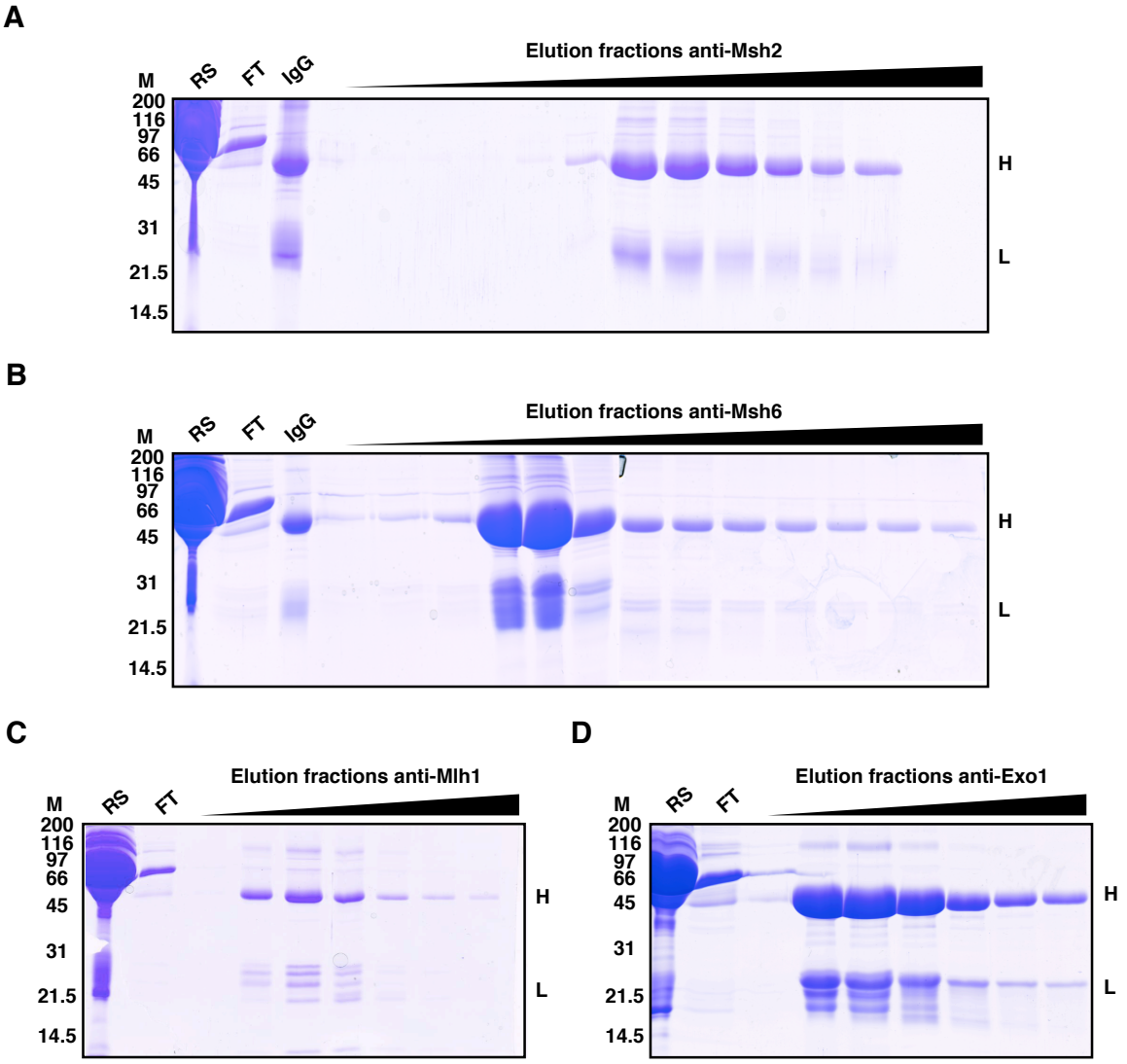


Fig. 15. Purification of rabbit antibodies raised against *Xenopus* MMR proteins. Coomassie staining showing samples taken during the purification of *Xenopus* MMR antibodies resolved on 12% SDS-PAGE. > 3 mg of protein fragments were coupled to Hi-trap NHS-activated HP and used as bait to capture specific immunoglobulins from the rabbit serum. RS, immunised rabbit serum diluted 1:10; FT, flowthrough obtained after binding of the serum to the "baited" column; IgG, 2 μg of reference antibody; black triangle, elution fraction obtained during the purification. H, IgG heavy chain, 55 kDa; L; IgG light chain, 25 kDa. M, size marker in kDa (Biorad broad range protein marker). A) Purification of anti-Msh2. B) Purification of anti-Msh6. C) Purification of anti-Mlh1. D) Purification of anti-Exo1.

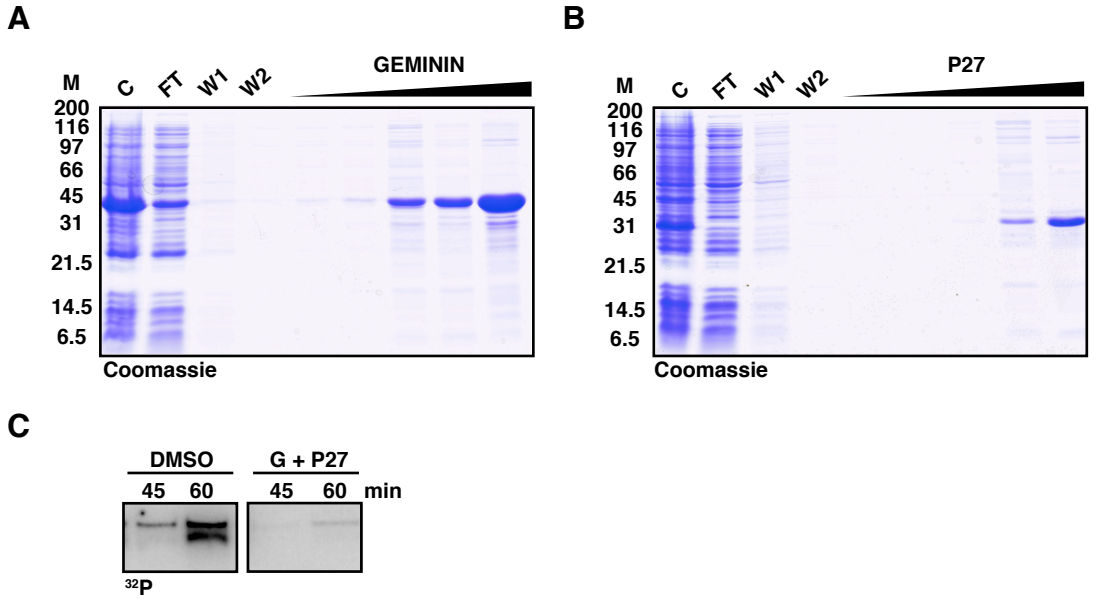


Fig. 16. Purification of replication inhibitors for the *Xenopus* egg extract system. A, B) Ni-NTA purified full-length human Geminin (25 kDa) and p27 (27 kDa). Constructs were cloned in pET28 and pET21, respectively. C) Inhibition of replication in XEE by recombinant Geminin (G) and p27 (P27) used at 500 nM. Replication products were visualized by incorporation of radioactive [α-^{32}P]dCTP (^{32}P). Constructs were a gift from the Costanzo Lab.

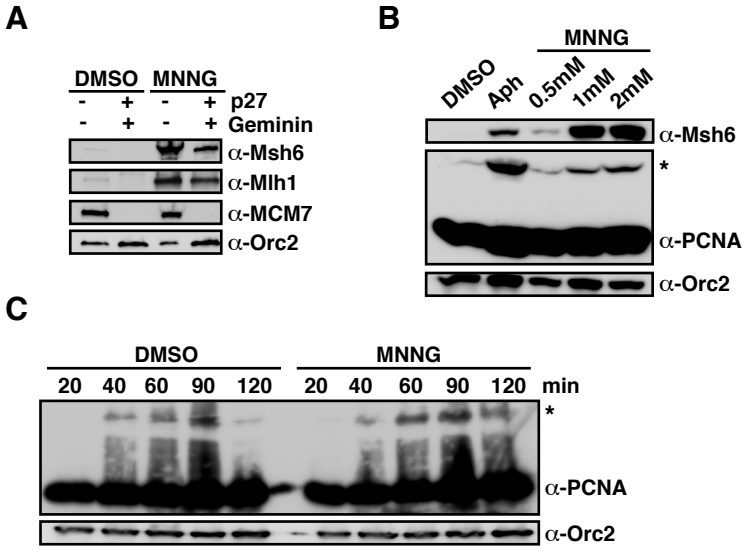


Fig. 17. Non-canonical MMR in S phase extract. A) Recruitment of xMMR proteins to damaged chromatin outside of replication as seen by addition of replication inhibitors Geminin and p27 to the reaction. B) Isolation of nuclei and chromatin-bound proteins during replication with different damaged templates (DMSO, undamaged control) shows that Msh6 recruitment to MNNG-damaged sperm chromatin is accompanied by ubiquitination of PCNA in the extract (*). C) Time course of replication of damaged (MNNG) and undamaged sperm chromatin shows persistent PCNA-Ub after replication of the damaged template.

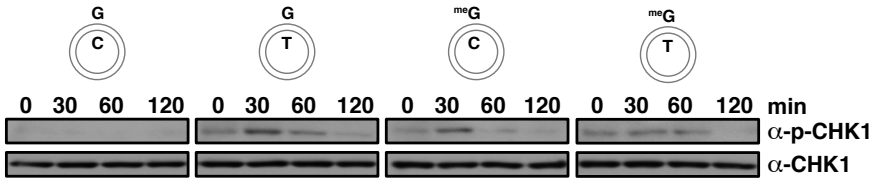


Fig. 18. Kinetic analysis of checkpoint activation (p-CHK1) during substrate replication. Indicated substrates (C/G, T/G, ^{me}G/C and ^{me}G/T) were replicated in HSS+NPE, samples were removed at the indicated times, resolved on a 7.5% SDS-PAGE and probed for p-CHK1 (S345) and CHK1. Time point 0 indicates addition of NPE to the HSS+DNA mix.

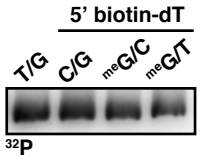


Fig. 19. Replication of biotinylated xMMR substrates. pRichi2850t substrates containing a single biotin-dT (see Materials and Methods for primer sequence) were replicated in HSS+NPE alongside a T/G substrate in the presence of [α-^{32}P]dCTP (^{32}P). Samples were removed 120 min after replication start, run on a 0.8% agarose gel and analyzed by autoradiography.

5.2.1 Materials and methods

Production of recombinant proteins for rabbit immunisation and for antibody purification

Fragments of *Xenopus* MMR proteins and full-length xMGMT were PCR amplified from EST clones obtained from Imagenes, using primers containing restriction sites for Nco1 and Xho1. The PCR products were then cloned in a pET28b(+) vector (Novagen). 6xHis-tagged protein fragments (Msh2F1: 258-404aa, Mlh1F1: 615-744aa, Msh6F1: 128-249aa, Exo1F1: 344-450 aa) were expressed in BL21 cells (Invitrogen) and purified on Ni-NTA agarose columns (Qiagen). Briefly, protein expression was induced with 0.5 mM IPTG in cells grown at 37°C. The cell pellets were resuspended in 20 mM Tris-HCl pH 8, 300 mM KCl, 10% glycerol, 1 mM β-mercaptoethanol, 0.1% NP40 and PMSF for lysis. After cell disruption and centrifugation of cell debris and membranes, the soluble fraction containing 40 mM imidazole was loaded onto a nickel chelating column. Washing was done with 25 mM imidazole and elution with 150 mM-1 M imidazole. Fractions were pooled and dialysed against either 20 mM MOPS/100 mM NaCl pH 7.9 for immunisation or coupling buffer for antibody purification. 100-200 μg of protein were used per round of rabbit immunisation.

hGeminin-pET28 and hp27-pET21 constructs were a kind gift of Yoshi Hashimoto/Vincenzo Costanzo (Clare Hall Laboratories, UK). The proteins were expressed as above.

For denaturing conditions (neccessary for Mlh1F1 fragment), pellets were induced as described above, resuspended in lysis buffer and centrifuged for 15 min 4°C. The pellet containing insoluble Mlh1F1 was resuspended in denaturing buffer containing 6 M urea, 100 mM NaH_2PO_4 and 10 mM Tris-HCl and incubated for 1 h. The beads were equilibrated with 6 M urea-containing buffer and the solubilised fraction was loaded onto the nickel chelating column. Washes and elution included 6 M urea. The eluted protein was then dialysed against coupling buffer cotaining 4 M, 2 M, 1 M urea and finally, no urea. For immunisation purposes, the protein was dialysed against 20 mM MOPS/100 mM NaCl/1 M urea.

Purification of *Xenopus* antibodies raised against MMR proteins

Antibody purification was performed using HiTrap NHS-activated HP column for affinity antibody purification as per manufacturers' instructions (GE Healthcare). This is a pre-packed column of N-hydroxy-succinimide (NHS) cross-linked to HP Sepharose beads. NHS reacts with ligands containing amino groups to give a very stable amide linkage. >3 mg of antigen (*Xenopus* MMR fragments) were affinity purified and coupled to the column overnight at 4°C in coupling buffer (200 mM $NaHCO_3$, 500 mM NaCl). 5 ml of rabbit serum were diluted to 50 ml in PBS and loaded onto the same column overnight at 4°C. The column was washed with buffer containing 10 mM Tris-HCl pH 7.5 and 500 mM NaCl and the antibody was eluted with 100 mM glycine pH 2.25. The pH of the fractions was adjusted immediately with 1 M Tris-HCl pH 8 and the antibody was concentrated using Amicon Ultra centrifugal filters (Millipore). Concentration was measured using Nanodrop.

Primers used for biotinylated substrates

Biotin C/G SalI primer

5' CGCGATC^{bio}TGATCAGATCCAGACGTCTGTCGACGTTGGGAAGC 3'

Biotin ^{me}G/C and ^{me}G/T*-SalI primer

5' CGCGATC^{bio}TGATCAGATCCAGACGTCTGTC^{me}GACGTTGGGAAGC 3'

***** pRichi2850top *AclI* was used for this substrate to pair a T opposite the ^{me}G

^{bio}T stands for biotinylated T

5.3 Additional observations

5.3.1 Role of MutSβ in O6-methylguanine (^{me}G) recognition

The MutSβ heterodimer is composed of MSH2 and MSH3 and is believed to be present in cells in a ratio of 10:1 with regard to MutSα [320]. Its role in MMR is in the repair of insertion/deletion loops larger than 2 nucleotides but it has also been shown to bind to ICLs generated by cisplatin and psoralen and as a protein that participates in the cytotoxic response to 5-fluorouracil [188, 202, 321]. The possibility of it playing a role in ^{me}G recognition first presented itself when *Xenopus* MMR assays showed that immunodepletion of Msh6 from the extract did not completely abrogate repair activity on the ^{me}G/T containing substrate as it did with the ^{me}G/C substrate (Fig. 19). This result was perplexing since, to our knowledge, there is no description in literature of a protein other than MutSα that recognizes ^{me}G-containing mismatches. What had been previously reported is that both MutS heterodimers can have functional overlap in certain contexts [322]. The immunodepletion showed that Msh6 depletion did not completely deplete the extract of Msh2 so the possibility that residual MutSβ could potentially recognize ^{me}G/T was therefore explored further.

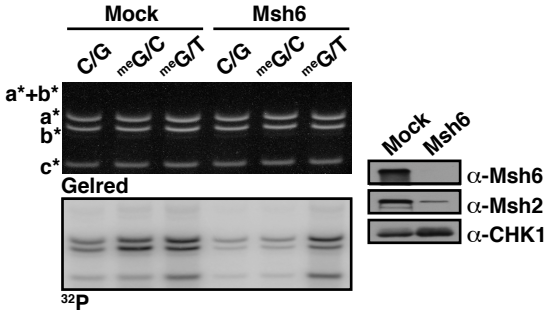


Fig 19. Recognition of ^{me}G substrates in *Xenopus* egg extract in Mock- and Msh6-depleted extract. Mock- and Msh6-depleted extracts were incubated with C/G, ^{me}G/C and ^{me}G/T pRichi2850t substrates for 30 min at 23°C under *Xenopus* MMR conditions (see 5.1, Materials and methods) and loaded on a 1% agarose gel after *HindIII*/*Dra*I digestion. Top: Digested samples analyzed on a 1% agarose gel and stained with GelRed. Restriction fragments are indicated by a*+b* (2484bp), a* (1336bp), b* (1148bp) and c* (694bp). Bottom: Autoradiograph (^{32}P) of the same agarose gel. Right, extract samples (Mock- and Msh6-depleted extract) were removed and subjected to blotting with the indicated antibodies.

In order to work with an *in vitro* system with previously characterized MMR deficiencies, we began working with nuclear extracts made from human LoVo cells, a colorectal cancer cell line which lacks both MSH3 and MSH6 and is therefore MMR deficient (Fig. 22A). Since both recombinant human MutSα as well as MutSβ were available, it was possible to selectively restore MMR with either heterodimer in a MMR assay. As expected, MutSα addition to the reaction restored repair activity to the T/G substrate and to a lesser extent the ^{me}G/T (Fig. 21A). Surprisingly, MutSβ displayed similar recognition capacity for ^{me}G/T. Indeed, when we performed a band shift assay for MMR susbtrates with recombinant MutSβ using a TTTT-loop containing substrate as a positive reference (Fig. 20) we could detect weak affinity of MutSβ for the ^{me}G substrates and not for the T/G substrate.

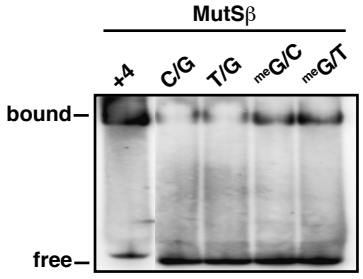


Fig 20. MutSβ binds to ^{me}G-containing mismatches. Band shift assay with recombinant human MutSβ (MSH2/MSH3) and 42mer heteroduplexes containing a TTTT loop (+4, positive control), C/G, T/G, ^{me}G/C or ^{me}G/T in their nucleotide sequence. The protein/DNA complexes were analyzed on a 5% native PAA (19:1) and visualized by autoradiography.

HCT15 is a colorectal cancer cell line where the cytotoxic response to the S_N1-type alkylator MNU was investigated [323]. Since it lacks MSH6 (Fig. 22A), it is known as a MMR deficient cell line and the focus of the study was to compare it to its MMR proficient counterpart, the HCT15 chromosome 2 cell line, where MSH6 was reintroduced. Interestingly, clonogenic assays performed in this study showed only a minor difference in response of two cell lines response to treatment with MNU; they were both sensitive to killing by this agent. We therefore used nuclear extracts from the HCT15 cell line and tested whether the MutSβ that is still expressed could be responsible for this sensitivity in a MMR assay (Fig. 21B). We included a looped substrate (+4) as a positive control for MutSβ and analyzed repair activity on T/G, ^{me}G/C and ^{me}G/T containing substrates. Here,

repair activity could be observed for all but the C/G control substrate. The looped substrate was the prefered one, followed by T/G, ^{me}G/T and slight ^{me}G/C recognition.

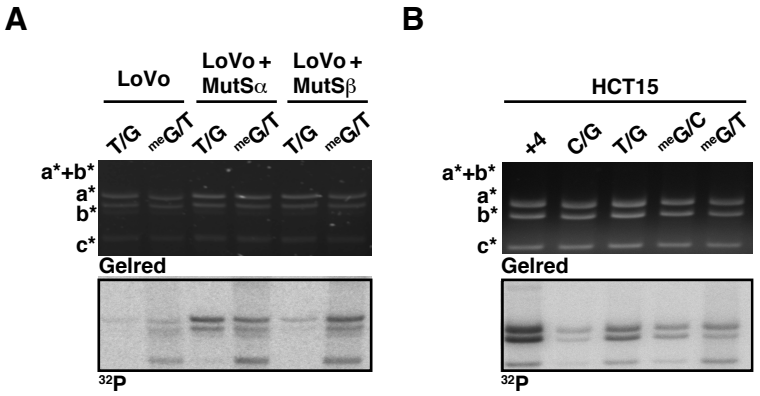


Fig. 21. MutSβ repair activity on ^{me}G/T pRichi2850t substrate in a MMR assay. A) The T/G and ^{me}G/T substrates were incubated with mismatch repair-deficient LoVo nuclear extracts (supplemented with recombinant MutSα (MSH2/MSH6) or MutSβ (MSH2/MSH3). Top, samples were loaded on a 1% GelRed-stained agarose gel after *HindIII*/*Dra*I digestion and repair activity was monitored by incorporation of [α-^{32}P]dCTP (^{32}P, bottom). B) A C/G control substrate and a substrate containing a TTTT loop (+4, positive control) were incubated alongside ^{me}G-containing substrates ^{me}G/C and ^{me}G/T in MutSα-deficient/MutSβ proficient HCT15 nuclear extracts. Top, samples were loaded on a 1% GelRed-stained agarose gel after *HindIII*/*Dra*I digestion and repair activity was monitored by incorporation of [α-^{32}P]dCTP (^{32}P, bottom).

Alltogether, there was a clear indication that MutSβ could recognize ^{me}G-mismatches *in vitro*, but we had to search for evidence that it could also do so *in vivo*. We turned to the human osteosarcoma U2OS cell line, which is widely used for a variety of cell-based assays, and set out to examine the effect of MSH3 downregulation in these cells and also ask if MutSβ is able to take over ^{me}G recognition when MSH6 is absent, using a chromatin binding assay. This would inform us as to whether MutSβ is recruited *in vivo* to ^{me}G lesions. We first confirmed efficient siRNA downregulation of both MSH6 and MSH3 via western blot (Fig. 22B). Both appeared fully downregulated. Unfortunately, MSH6 downregulation led to significant co-downregulation of MSH2, which could of course accentuate and mask the phenotype of only downregulating MSH6.

The chromatin binding assay in Fig. 22C shows recruitment of the proteins of interest in response to treatment of non-synchronised U2OS cells with 1 mM MNU. Cells were pretreated with O6-benzylguanine so that the recruitment shown was ^{me}G-dependent. Curiously, downregulation of MSH6 did not completely impair recruitment of MSH2 and

MLH1 to the chromatin even though it was substantially decreased as compared to the siLuc control samples. Whether this residual MSH2 heterodimerised with MSH3 and was recruited to ^{me}G is difficult to assess, since MSH3 is strongly present on the chromatin regardless of treatment. Interestingly, downregulation of MSH3 also led to decreased recruitment of MSH6 to chromatin.

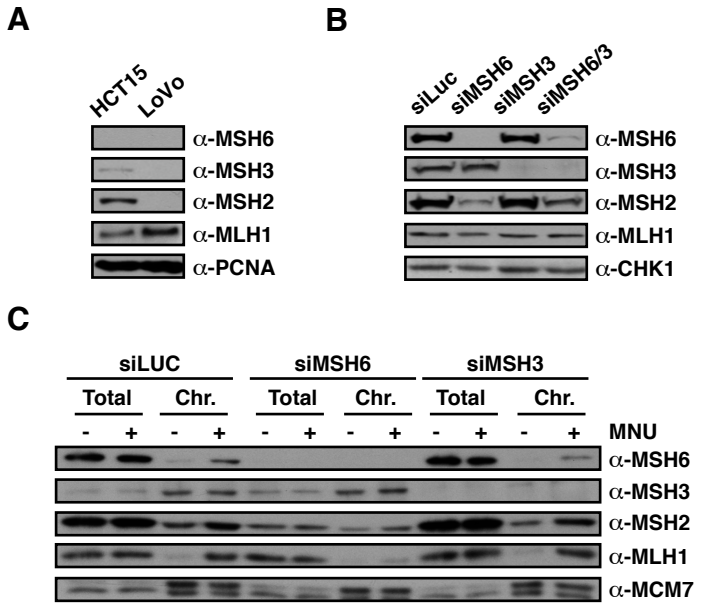


Fig. 22. MutSβ recognition of ^{me}G substrates *in vivo*. A) MMR status of human adenocarcinoma cell lines LoVo (mismatch repair deficient) and HCT15 (MSH6 deficient). 30 μg of nuclear extract were resolved on a 10% SDS-PAGE and subjected to western blotting with the indicated antibodies. B) MMR status of the human osteosarcoma cell line U2OS (mismatch repair proficient) after downregulation of MSH3 and MSH6. Western blot showing the efficiency of MSH3 downregulation by siRNA Smartpool in U2OS. MSH6 was downregulated using a single siRNA against it and siLuc served as control. Samples were harvested 72 hours after siRNA transfection and resolved on a 6% SDS-PAGE. C) Western blot of total- and chromatin-bound MMR proteins in MNU-treated U2OS cells (1 mM treatment 3 hours prior to harvesting).

These results are preliminary and conducted in this cell line only. It would be very interesting to see recruitment of MMR proteins in the HCT15 cell line which only expresses MSH3. Experiments are also currently under way to determine the right dose of MNU that can be used for similar chromatin binding assays as well as a clonogenic assay. Since it was already previously established that the HCT15 is not as resistant to MNU treatment as their MMR deficient cell line, it would be tempting to downregulate MSH3 in this cell line and observe whether that increases resistance to MNU.

5.3.2 Materials and methods

Substrates, nuclear extracts and MMR assays

The substrates and nuclear extracts were generated as described previously [316]. Briefly, the hetero- and homoduplexes were constructed by primer extension using the oligonucleotides listed below as primers and single-stranded phagemid DNA (pRichi2850top *Sal*I or *AclI*, creates 5' nicked substrates) as template. After primer extension, ligation and isolation of the desired supercoiled heteroduplex substrates on CsCl gradients, MMR assays were carried out as described [316].

Unless otherwise specified, the MMR reactions were carried out with 100 ng DNA substrate and 100 μg nuclear extracts in a total volume of 25 μl in a buffer containing 20 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$, 110 mM KCl, 1 mM glutathione, 50 μg/μl BSA, 100 mM dNTPs and, where indicated, 2 μCi of [α-^{32}P]dCTP (^{32}P). When indicated, recombinant human MutSα or MutSβ were added to the reaction. The extracts were incubated at 37°C for 40 min. The reaction was stopped by adding an equal volume of 2 x STOP solution containing 1 mM EDTA, 3% SDS and 5 mg/ml proteinase K. The samples were incubated at 45°C for 30 min, purified on Mini-Clean columns (Qiagen) and subjected to restriction digests. The digested DNA was resolved on 1% agarose gels.

Primers for substrates and the band shift assay

C/G-*Sal*I primer:

5' CCAGACGTCTGT**C**GACGTTGGGAAGCTTGAG 3'

T/G-*Sal*I primer:

5' CCAGACGTCTGT**T**GACGTTGGGAAGCTTGAG 3'

+4-*Sal*I primer:

5' CCAGACGTCTGTC**TTTT**GACGTTGGGAAGCTTGAG 3'

^{me}G/C and ^{me}G/T -SalI primer:

5' CCAGACGTCTGTC^{me}**G**ACGTTGGGAAGCTTGAG 3'

***** pRichi2850top *AclI* was used for this substrate to pair a T opposite the ^{me}G

Band shift assay

This was performed essentially as described [208]. The oligonucleotide heteroduplexes were created by annealing 5' radiolabelled (*) oligonucleotides (see **Primers for substrates and the band shift assay**) with unlabeled complementary oligonucleotides. The binding reaction mixtures contained 40 fmol heteroduplex with a +4, C/G, ^{me}G/C or ^{me}G/T mismatch and recombinant human MSH2/MSH3 (250 ng). Protein-bound substrates were separated from free probes by electrophoresis on a 5% native polyacrylamide gel with TAE.

Antibodies

Antibodies against MSH6 (BD Transduction Laboratories), MSH2 (Calbiochem), MLH1 (BD Pharmigen), CHK1 (Santa Cruz), PCNA (Santa Cruz) and MCM7 (Sigma) were obtained commercially. The antibody against MSH3 was a kind gift from the IMCR.

siRNA and knockdown experiments

U2OS cells were transfected using 40nM siRNA against luciferase (siLuc), MSH6 (Microsynth), MSH3 (Smartpool from Thermo Scientific) or both, using RNAiMax (Invitrogen) as per the manufacturer's protocol. 72 hours post-transfection, total cell extracts were prepared using Laemmli buffer (120 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS). The siRNAs used in this study were as follows: siLuc: 5' CGU ACG CGG AAU ACU UCG A 3', siMSH3.1: 5' GCA CAU AGC UAC AGA AAU U 3', siMSH3.2: 5' CCC GAG AGC UCA AUA UUU A 3', siMSH3.3: 5' GGA CAG GAG UUU AUG AUA G 3', siMSH3.4: 5' GAU UCG AAA CGU CAA AUU A 3', siMSH6: 5' CGC CAT TGT TCG AGA TTT A 3'

Triton extraction for chromatin binding

To isolate the Triton-insoluble fraction, cells were rinsed twice in cold PBS, incubated for 10 min on ice in preextraction buffer (25 mM Hepes-KOH pH 7.4, 50 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$, 300 mM sucrose, 0.5% Triton X-100 and protease inhibitors). After removal of buffer and rinsing in PBS, adhering cellular material (Triton-insoluble fraction) was harvested by scraping the cells into Laemmli buffer (4% SDS, 20% glycerol and 120 mM Tris-HCl pH 6.8). The enriched chromatin fraction was then heat denatured, fragmented by syringe and analyzed by western blotting.

5.4 Ribonucleotides misincorporated into DNA act as strand-discrimination signals in eukaryotic mismatch repair

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This manuscript investigated the possibility of falsely incorporated ribonucleotides acting as strand discrimination signals for MMR during replication. It had recently been discovered that replicative polymerases, specially the leading strand polmerase pol ε, can misincorporate ribonucleotides into DNA and that a pathway now known as ribonucleotide excision repair (RER) removes these from our DNA. It is largely assumed that Okazaki fragments signal discontinuities in DNA that MMR uses to distinguish the parental strand from the daughter strand during replication. When it comes to the leading strand, however, how this occurs remained puzzling and it is only now that the field is becoming open to the possibility of MMR hijacking the repair of other lesions and using this as a strand discrimination signal. Ribonucleotide repair, because of its relatively abundant activity on the leading strand during replication, seemed a likely candidate. To study this, established biochemical assays for MMR activity as well as yeast reporter assays to confirm the hypothesis *in vivo* were used. These sets of experiments demonstrated that indeed, errors ocurring during replication of the leading strand can provide additional entry points for MMR, thereby increasing genome fidelity.

I contributed to this study by designing the phagemid used for substrate preparation, extract preparation, performing the band shift with recombinant Mutsα which showed that it does not recognize rC/G mismatches as substrates (Fig. 1G), helping design the model (Fig. 4) as well as by critically reading the manuscript.

Please cite this article in press as: Ghodgaonkar et al., Ribonucleotides Misincorporated into DNA Act as Strand-Discrimination Signals in Eukaryotic Mismatch Repair, Molecular Cell (2013), http://dx.doi.org/10.1016/j.molcel.2013.03.019

Molecular Cell
Article

Ribonucleotides Misincorporated into DNA Act as Strand-Discrimination Signals in Eukaryotic Mismatch Repair

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http://dx.doi.org/10.1016/j.molcel.2013.03.019

SUMMARY

To improve replication fidelity, mismatch repair (MMR) must detect non-Watson-Crick base pairs and direct their repair to the nascent DNA strand. Eukaryotic MMR in vitro requires pre-existing strand discontinuities for initiation; consequently, it has been postulated that MMR in vivo initiates at Okazaki fragment termini in the lagging strand and at nicks generated in the leading strand by the mismatch-activated MLH1/PMS2 endonuclease. We now show that a single ribonucleotide in the vicinity of a mismatch can act as an initiation site for MMR in human cell extracts and that MMR activation in this system is dependent on RNase H2. As loss of RNase H2 in *S.cerevisiae* results in a mild MMR defect that is reflected in increased mutagenesis, MMR in vivo might also initiate at RNase H2-generated nicks. We therefore propose that ribonucleotides misincorporated during DNA replication serve as physiological markers of the nascent DNA strand.

INTRODUCTION

Correction of replication errors requires that MMR be targeted to the nascent DNA strand. In gram-negative bacteria such as *Escherichia coli* (*E. coli*), newly synthesized DNA is transiently unmethylated at adenines in d(GATC) sites, and this allows the MutS/MutL-activated MutH endonuclease to introduce a nick into the undermethylated nascent strand, where exonucleolytic degradation of the error-containing section commences (Jiricny, 2006). Gram-positive bacteria and eukaryotes do not use methylation in strand discrimination, and it was suggested that MMR may be directed to nascent DNA by strand discontinuities such as gaps between Okazaki fragments (Claverys and Lacks, 1986). This hypothesis was supported experimentally in extracts of human and *D. melanogaster* cells, where a single nick was shown to be necessary and sufficient to direct MMR to the discontinuous strand of a circular heteroduplex substrate carrying a single mismatch (Holmes et al., 1990; Thomas et al., 1991). The repair process could be directed by nicks situated either 3′ or 5′ from the misincorporated nucleotide. This was puzzling, because EXO1, the only exonuclease implicated in eukaryotic MMR, has an obligate 5′ → 3′ polarity. An answer to this puzzle came when the PMS2 subunit of MutLα (a heterodimer of MMR proteins MLH1 and PMS2) was shown to associate with PCNA and introduce additional nicks into the discontinuous strand that could be used as EXO1 loading sites (Pluciennik et al., 2010). This finding helped explain how MMR directs the repair process into the nascent DNA in both lagging and leading strands (Peña-Diaz and Jiricny, 2010). However, on the leading strand, the MutLα/PCNA complex would have to travel from the 3′ terminus toward and past the mismatch that could be several hundred nucleotides distant, and also the MMR system would have to compete with nucleosome loading behind the replication fork (Schöpf et al., 2012). Therefore, it was anticipated that MMR on the leading strand might be less efficient than on the lagging strand, where strand discontinuities are readily available. This was indeed shown to be the case (Nick McElhinny et al., 2010a), but because the difference was relatively small, it was postulated that MMR efficiency in the leading strand might be augmented by additional factors.

Recently, more than a million ribonucleotide monophosphates (rNMPs) were reported to be incorporated into mouse genomic DNA during replication (Hiller et al., 2012; Reijns et al., 2012), and a similar situation exists in *Saccharomyces cerevisiae* (*S. cerevisiae*), where the leading-strand polymerase (pol-ε) incorporates approximately four times more ribonucleotides into the nascent DNA than the lagging-strand enzyme (pol-δ): 1 rNMP/1,250 dNMPs versus 1/5,000, respectively (Nick McElhinny et al., 2010b, 2010c).

Ribonucleotides are removed from DNA by RNases H1 and H2, whereby the former enzyme processes stretches of more than three rNMPs such as those found at the 5′ termini of Okazaki fragments, while RNase H2 can incise the hybrid strand 5′ from even a single rNMP (Eder and Walder, 1991). The ribonucleotide(s) can then be removed by the flap endonuclease FEN1 and/or EXO1 (Rydberg and Game, 2002; Sparks et al., 2012). We wondered whether RNase H2-generated strand breaks arising during rNMP removal might be utilized by the MMR system as initiation sites for the exonucleolytic degradation of the

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Ribonucleotides as MMR Initiation Sites

error-containing strand. Here, we show that a single ribonucleotide in close proximity of a mismatch can act as an initiation site for MMR in cell extracts as well as in a reconstituted system, and that MMR activation in this scenario is dependent on RNase H2. As a consequence, loss of RNase H2 in *S. cerevisiae* leads to a mild defect in MMR and increased mutagenesis.

RESULTS

We first made use of an in vitro MMR assay (Baerenfaller et al., 2006; Modrich, 2006; Schanz et al., 2009), in which a phagemid substrate containing a T/G mismatch in a SalI restriction site is incubated with extracts of human cells. Because MMR requires a pre-existing strand discontinuity for initiation, the T/G mispair in a closed circular or supercoiled (sc) substrate is not repaired. However, introduction of an *Nt.Bst*NBI-catalyzed nick 361 nucleotides 5′ from the mispaired T licenses MMR, which corrects the T/G mismatch to C/G and thus makes the phagemid susceptible to SalI digestion. In this system, MMR efficiency is estimated by digesting the phagemid recovered from the extracts with SalI and DraI, and quantitating the fraction of DNA in the 1,324 (band **a**) and 1,160 (band **b**) base-pair fragments, which are indicative of successful MMR (Figure 1A).

Ribonucleotide-Dependent Mismatch Repair in Human Cell-free Extracts

We first generated a substrate containing a single rCMP residue (rC) 54 nucleotides 5′ from the mispaired T (rC-T/G), as well as a homoduplex (rC-C/G) control, and incubated them with extracts of LoVo cells, which lack the major mismatch recognition factor MSH2/MSH6 (MutSα) and are thus MMR deficient. As anticipated, none of the heteroduplex substrates were repaired (Figure 1B, lanes 5–8), but when the extract was supplemented with purified recombinant MutSα, ~60% of the *Nt.Bst*NBI-nicked T/G substrate was corrected to C/G within 30 min (Figure 1B, lane 2). The supercoiled T/G substrate remained largely uncorrected (Figure 1B, lane 1). Importantly, the supercoiled rC-T/G substrate was repaired nearly as efficiently as the nicked T/G phagemid in a reaction dependent on MutSα (Figure 1B, lane 3, compare with lane 2), and the efficiency of repair was only slightly improved by *Nt.Bst*NBI pretreatment (Figure 1B, lane 4). Kinetic analysis (Figure 1C) further confirmed that a single ribonucleotide in the supercoiled T/G heteroduplex was sufficient to convert a MMR-refractory substrate into a MMR-susceptible one in this system.

We repeated the above experiments with MMR-deficient 293TLα extracts (293T) that lack MutLα (Cejka et al., 2003). When these extracts were supplemented with purified recombinant MutLα, the supercoiled rC-T/G substrate was repaired almost as efficiently as the nicked rC-T/G or T/G heteroduplexes (Figure 1D, lanes 2–4). As observed in previous studies (Constantin et al., 2005; Schanz et al., 2009), small amounts of repair were detected also in the absence of added MutLα, as this factor is not absolutely required for 5′ → 3′ MMR (Figure 1D, lanes 5–8). When compared with MutSα-supplemented LoVo extracts (Figure 1B, lane 1; and Figure 1C), the supercoiled T/G substrate was repaired to a greater extent in the MutLα-supplemented 293T extracts (Figure 1D, lane 1; and Figure 1E). This is most likely due to the endonuclease activity of MutLα, which can introduce random nicks with low efficiency also into either strand of a supercoiled substrate (Pluciennik et al., 2010) and trigger noncanonical MMR (Peña-Diaz et al., 2012).

By carrying out the in vitro MMR reactions in the presence of [α-^{32}P]dCTP, we saw only background levels of radioactivity incorporated into the T/G or rC-T/G substrates incubated with LoVo extracts in the absence of added MutSα (Figure 1B, lanes 5–8 in lower panel). Similarly, the rC-C/G control substrate remained largely unlabeled even when the extracts were supplemented with MutSα (lanes 9–12 in lower panel). This shows that the mere presence of the ribonucleotide in a substrate does not trigger long-patch DNA synthesis, irrespective of whether the extracts are MMR deficient or proficient.

Identical experiments carried out in the MMR-deficient extracts of 293TLα cells yielded similar results, even though the background levels of [α-^{32}P]dCMP incorporation into the T/G and rC-T/G substrates were somewhat higher (Figure 1D, lanes 5–8 in lower panel; compare with Figure 1B, lanes 5–8 in lower panel). This is due to residual 5′ → 3′ MMR that can proceed independently of MutLα, and that initiates at rare random nicks in the closed circular phagemids. Notably, the **a** + **b** fragments contained proportionally more radioactive nucleotide than the cleaved fragments **a** and **b**. This indicates that most of the excision/resynthesis events failed to reach the mispaired T and therefore convert the T/G mispair to C/G, which would have restored the SalI site.

In contrast to 5′ → 3′ repair, MutLα is indispensable for 3′ → 5′ MMR (Kadyrov et al., 2006). To learn whether similar criteria apply also to ribonucleotide-directed MMR, we first constructed a substrate that contained the rCMP residue 60 or 308 nucleotides 3′ from the mispaired T (T/G-rC). When incubated with 293TLα extracts that are devoid of MutLα, no repair of the control T/G phagemid was detected, irrespective of whether the substrate was supercoiled or nicked (Figure 1F, lanes 1 and 2, respectively). This result differed from that obtained with the 5′-nicked substrate (Figure 1D, lane 6) and confirms the requirement for MutLα in 3′ nick-directed MMR (Pluciennik et al., 2010). Similarly, only background levels of [α-^{32}P]dCMP were incorporated into the T/G-rC substrates (Figure 1F, lanes 3 and 4). However, when the extracts were supplemented with purified recombinant MutLα, the closed circular T/G-rC substrates were repaired almost as efficiently as the nicked T/G heteroduplex (Figure 1F, compare lane 6 with lane 7 or lane 8).

Taken together, the above results demonstrate that strand discontinuities arising during processing of ribonucleotides in DNA can be hijacked by the MMR machinery for initiation of mismatch-activated excision, even over a distance of more than 300 nucleotides. Moreover, the finding that only background levels of [α-^{32}P]dCMP were incorporated into the rC-C/G phagemid (Figure 1B, lanes 9–12) showed that removal of the ribonucleotide, presumably by an RNaseH2-dependent mechanism (Sparks et al., 2012), involved only short-patch (fewer than ~30 nucleotides) repair synthesis. The mere presence of the ribonucleotide in these substrates was not sufficient to trigger long-patch repair synthesis, be it MMR independent or MMR dependent. The lack of activation of the MMR process by the rCMP was further strengthened by the finding that purified

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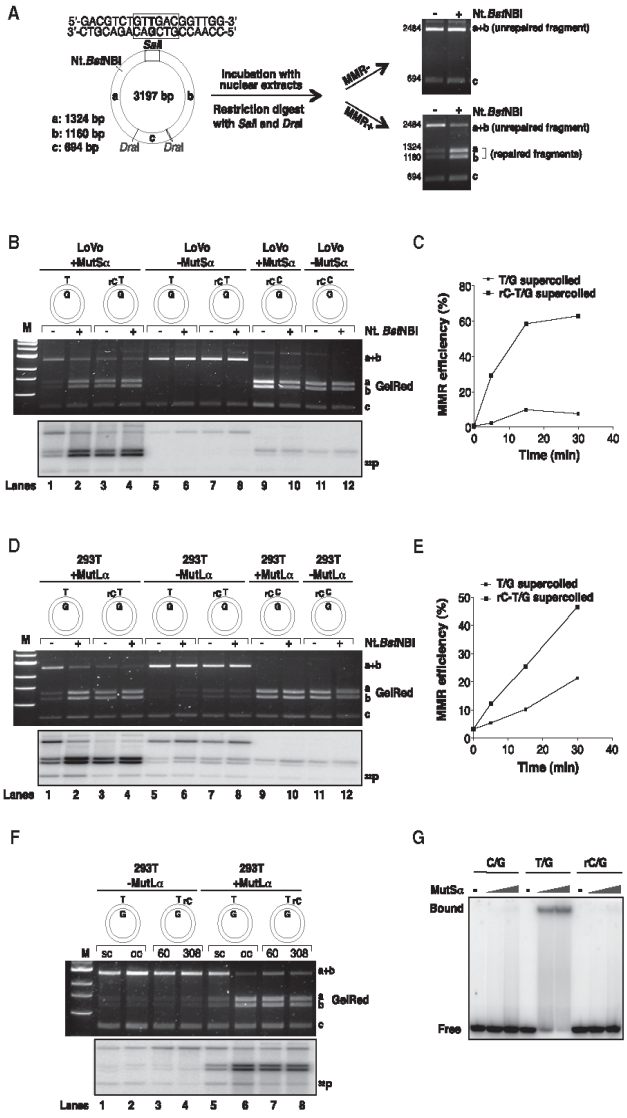

Figure 1. A Single Ribonucleotide in a DNA Heteroduplex Acts as an Initiation Site for MMR

(A) Schematic representation of the in vitro MMR assay. In the absence of a nick, very little repair of the T/G mismatch takes place, and digestion of the phagemid DNA with SalI and DraI gives rise to 2,484 (**a** + **b**), 694 (**c**), and 19 bp fragments (the smallest fragment is not detectable on these 1% agarose gels stained with GelRed). Upon introduction of a nick at the *Nt.Bst*NBI site, T/G-to-C/G repair restores the SalI site, such that the phagemid DNA is cut into 1,324 (**a**), 1,160 (**b**), 694 (**c**), and 19 bp fragments.

(B) The presence of a single cytidine (rC) 54 nucleotides 5′ from the mispaired T made the T/G substrate susceptible to MMR even in the absence of a *Nt.Bst*NBI nick. The substrates were incubated with MutSα-deficient LoVo nuclear extracts (supplemented or not with recombinant MutSα), and repair efficiency was quantitated by estimating the percentage of DNA in bands **a** and **b**. Autoradiograph of the same gel (^{32}P) showed that [α-^{32}P]dCMP was incorporated preferentially into the repaired fragments **a** and **b**. The figure shows a representative result. The experiment was carried out in triplicate.

(C) Kinetic analysis performed with the supercoiled T/G and rC-T/G substrates in MutSα-complemented LoVo extracts.

(D) As in (B), but the substrates were incubated with MutLα-deficient extracts of 293TLα cells supplemented or not with recombinant MutLα.

(E) Kinetic analysis performed with the supercoiled T/G and rC-T/G substrates in MutLα-complemented 293TLα extracts.

(F) Position and distance of the ribocytidine does not affect MMR efficiency. Supercoiled (sc), *Nt.Bst*NBI-nicked (oc) T/G phagemids, or T/G-rC substrates containing a single rC residue 60 or 308 nucleotides 3′ from the mispaired T, were incubated with 293TLα cells supplemented or not with recombinant MutLα. Repair efficiency was quantitated by estimating the percentage of DNA in bands **a** and **b**. Autoradiograph of the same gel (^{32}P) showed that [α-^{32}P]dCMP was incorporated preferentially into the repaired fragments **a** and **b**. The figure shows a representative result.

(G) Band-shift assay (Cejka et al., 2005) using recombinant MutSα and oligonucleotides C/G, T/G, or rC/G. The figure shows an autoradiograph of a 6% native polyacrylamide gel. M, size marker (1 kb ladder, New England BioLabs).

recombinant human MutSα did not bind the rC/G "mispair" in an electrophoretic mobility-shift assay (Figure 1G), similarly to *S. cerevisiae* MutSα, which bound even an rG/T mispair with only very low affinity (Clark et al., 2011).

RNase H2, but Not H1, Can Initiate Mismatch-Dependent Strand Degradation in a Reconstituted In Vitro System

We wanted to learn which enzyme was responsible for incising the heteroduplexes in the above in vitro MMR assays. Since ribonucleotide removal from DNA is initiated by RNase H, we tested the activity of RNases H1 and H2 on our substrate. Incubation of the rC-T/G and rC-C/G substrates with bacterial RNase HII or with human RNase H2 led to the formation of open-circular forms of the phagemids, whereas those incubated with bacterial RNase HI or a catalytically inactive version of human RNase H2 (D34A/D169A or DD/AA) remained supercoiled (Figures 2A and 2B). This result agrees with work from other laboratories, which reported that only RNase H2 can incise RNA/DNA hybrids

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Ribonucleotides as MMR Initiation Sites

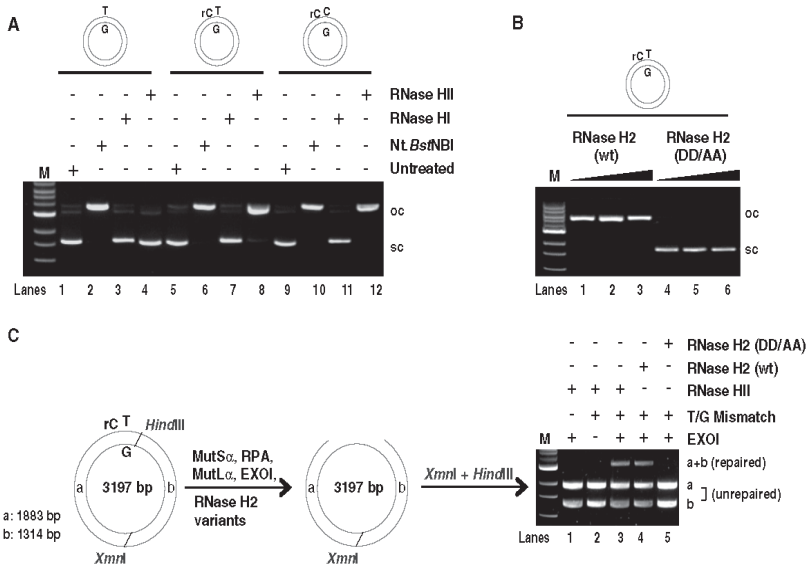

Figure 2. RNase H2-Mediated Processing of a Single Ribonucleotide in the Heteroduplex Substrate Provides MMR with an Initiation Site In Vitro

(A and B) The indicated supercoiled (sc) substrates were incubated with (A) purified recombinant RNase HI or HII or with the nickase *Nt.Bst*NBI or (B) increasing concentrations of human recombinant RNase H2 wild-type (WT) or the catalytically inactive (DD/AA) mutant. Only RNase HII, *Nt.Bst*NBI, and RNase H2 WT generated open circular (oc) form, which confirms that only RNase H type 2 ribonuclease can incise DNA substrates containing a single ribonucleotide.

(C) Schematic representation of mismatch-dependent strand-degradation reaction, using the indicated purified recombinant proteins. Phagemid molecules containing MMR-generated single-stranded gaps around the mismatch (indicative of efficient strand degradation) are resistant to cleavage with HindIII and are only linearized with XmnI (**a** + **b**). Fully double-stranded (unrepaired) molecules are cleaved with both enzymes into fragments **a** and **b**. M, size marker (1 kb ladder, New England BioLabs).

containing single ribonucleotides (Cerritelli and Crouch, 2009; Reijns et al., 2012; Sparks et al., 2012).

We wanted to confirm our findings by reconstituting the ribonucleotide-dependent MMR reaction from individual purified proteins. Using a substrate containing a nick within ~1 kb of the mispair, the minimal 5′-to-3′ MMR system could be reconstituted from MutSα, MutLα, RPA, and EXO1 (Genschel and Modrich, 2003). In that system, mismatch-dependent strand degradation gave rise to a single-stranded gap spanning the distance between the nick and ~150 nucleotides past the mismatch. The appearance of the single-stranded gap could be monitored by restriction enzyme digestion, because the substrate became refractory to cleavage by enzymes with recognition sites within the gap. We decided to adopt this approach but substitute the T/G substrate with the rC-C/G or rC-T/G phagemids, which can be digested with HindIII and XmnI to give rise to fragments **a** and **b** of 1,883 and 1,314 bp, respectively. The HindIII recognition sequence is situated ten base pairs from the T/G mismatch and is thus an ideal indicator of single-stranded gap formation in this region. As shown in Figure 2C, incubation of the rC-C/G control substrate with MutSα, MutLα, RPA, EXO1, and bacterial RNase HII, followed by digestion with HindIII and XmnI, yielded only the fragments **a** and **b** (lane 1). A similar result was obtained with the rC-T/G substrate when EXO1 was omitted (lane 2). In contrast, incubation of the rC-T/G substrate with MutSα, MutLα, RPA, EXO1, and bacterial RNase HII (lane 3) or human RNase H2 (lane 4) gave rise, in addition to fragments **a** and **b**, also to fragment **a** + **b**, which is indicative of single-strand gap formation. Addition of the RNase H2 DD/AA mutant (lane 5) yielded no **a** + **b** fragment. Collectively, these data provided formal proof that mismatch- and MutSα/MutLα-activated EXO1 could load at RNase H2-catalyzed strand breaks and initiate the strand-degradation process in spite of the fact that the 5′ terminus of the nick is a ribonucleotide.

RNase H2-Mediated MMR in Human and Mouse Nuclear Extracts

We next set out to confirm that the MMR process seen in nuclear extracts was also dependent on RNase H2. In the first experiment, we knocked down RNase H1, RNASEH2A, or both in 293 cells by siRNA (Figure 3A, bottom right panel). The knockdown of RNASEH2A decreased repair efficiency of the rC-T/G substrate to ~50% of that seen in extracts of cells treated with siLUC (Figure 3A, compare lanes 5 and 6), while knockdown of RNase H1 had no apparent effect (Figure 3A, lanes 3 and 7). We next immunodepleted RNase H2 from MutLα-deficient

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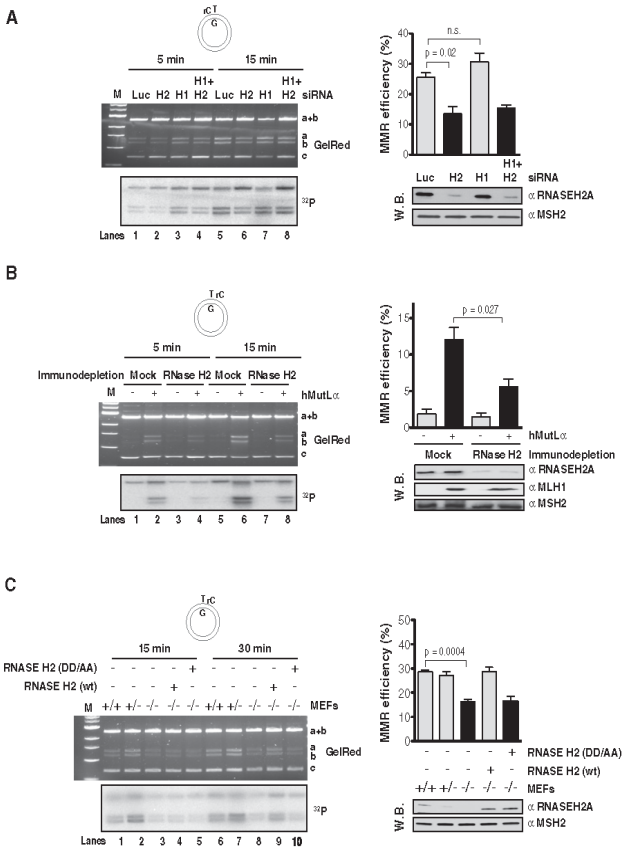

Figure 3. RNase H2-Activated MMR in Human and Mouse Nuclear Extracts

(A) RNASEH2A knockdown decreases MMR activity on the rC-T/G heteroduplex. (Left panels) Efficiency of the mismatch repair reaction at 5 and 15 min time points in extracts of 293 cells transiently transfected with the indicated siRNAs. The figure shows an agarose gel image (GelRed) and autoradiograph (^{32}P) of a representative experiment. (Top right) Plot of data from the 15 min time points of three independent experiments, with error bars representing standard deviation from the mean. (Bottom right) Western blot showing siRNA-mediated knockdown efficiency of RNASEH2A in 293 cells. Luc, siRNA against luciferase control; H1, H2a, H1+H2a, siRNAs against RNase H1, H2a, or both.

(B) Immunodepletion of RNase H2 decreases MMR activity on the T/G-rC heteroduplex. (Left panels) MMR assay carried out in extracts of 293T cells immunodepleted of RNase H2. The figure shows an agarose gel image (GelRed) and autoradiograph (^{32}P) of 5 and 15 min time points of a representative experiment. (Top right) Plot of data from the 15 min time points of three independent experiments, with error bars representing standard deviation from the mean. (Bottom right) Immunodepletion of RNase H2 and supplementation of 293T extracts with recombinant MutLα. This western blot shows the efficiency of the immunodepletion procedure and the amounts of recombinant MLH1 relative to endogenous MSH2 levels.

(C) Extracts of RNase H2 knockout mouse embryonal fibroblasts display decreased MMR activity on the T/G-rC heteroduplex at the 15 and 30 min time points. (Left panel) An agarose gel image (GelRed) and autoradiograph (^{32}P) of a representative experiment. (Top right) Plot of data from the 30 min time points of three independent experiments, with error bars representing standard deviation from the mean. (Bottom right) Western blot showing the amount of RNASEH2A in the MEFs as well as the amount of recombinant RNase H2 protein added to restore the MMR defect. MSH2 visualized with an anti-MSH2 antibody served as the loading control in the above experiments. M, size marker (1 kb ladder, New England BioLabs).

293T nuclear extracts, using a polyclonal antibody raised against the heterotrimer (Figure 3A, bottom right). As anticipated, no repair activity was observed in the absence of MutLα in either mock- or RNase H2-depleted extracts (Figure 3B, lanes 1, 3, 5, and 7), whereas addition of recombinant MutLα successfully rescued MMR in the mock-depleted extracts (lanes 2 and 6). In contrast, MutLα rescued MMR only weakly in the RNase H2-depleted extracts (lanes 4 and 8). Finally, we employed nuclear extracts derived from RNase H2 WT (+/+), heterozygous (+/−), and homozygous null (−/−) mouse fibroblasts (Reijns et al., 2012) and performed MMR assays with the T/G-rC substrate (Figure 3C, bottom right). As shown in Figure 3C, there was no significant difference in repair efficiency between WT and heterozygous fibroblasts (compare lanes 2 and 7 with lanes 1 and 6). However, RNase H2 deficiency resulted in a substantial decrease in repair efficiency when compared to WT extracts (compare lanes 3 and 8 with lanes 1 and 6). Importantly, this MMR defect could be completely rescued by the addition of recombinant human WT RNase H2 (lane 9), but not the DD/AA mutant (lane 10). Our data therefore show that RNase H2 is the key nuclease responsible for MMR initiation in the rC-T/G substrate in human and mouse cell extracts.

Loss of RNase H2 in *S. cerevisiae* Impairs MMR in the Leading Strand

In the above experiments we have demonstrated that RNase H2 can direct MMR to the ribonucleotide-containing strand and thus facilitate strand discrimination in MMR assays using cell extracts. We next wanted to test whether RNase H2 performs this function also in vivo. To this point, we made use of an assay in which the fidelity of leading- and lagging-strand replication can be monitored by the reversion rate of a mutant *ura3-29* allele at the *agp1* locus of a *S. cerevisiae ogg1Δ* strain. In this genetic background, reversion of the *ura3-29* point mutation was

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Table 1. Reversion Rates of a Mutant *ura3-29* Allele at the *agp1* Locus of a *S. cerevisiae ogg1Δ* Strain

Strain	*ura3-29* Orientation	Mutation Rate × 10^{-7}	95% Confidence Limits	Fold Increase Relative to WT	Residual MMR Activity (%)
Wild-type	LD	3.95	(3.25–4.4)	1	100
	LG	1.01	(0.69–1.25)	1	100
rnh201Δ	LD	5.86	(4.91–8.47)	1.48	76
	LG	1.64	(1.41–1.93)	1.62	90
msh2Δ	LD	12.05	(9.89–13.25)	3.05	0
	LG	6.95	(5.97–8.14)	6.89	0
msh2Δrnh201Δ	LD	11.75	(10.41–12.61)	2.98	4
	LG	7.16	(5.28–9.12)	7.09	2

LD represents the leading strand and LG the lagging strand. Data from one representative experiment are shown. Fold increase was calculated relative to the wild type (WT) strain. The residual MMR activity was calculated using the following formula: [1 − (mutation rate of mutant strain − mutation rate of WT)/(mutation rate of *msh2Δ* strain − mutation rate of WT)] × 100, as described previously (Pavlov et al., 2003).

previously shown to be caused by MMR dysfunction (Pavlov et al., 2003), whereby the loss of *MSH2* increased lagging-strand (OR2 in Table 1) mutagenesis nearly 7-fold, as compared to only 3-fold in the leading strand (OR1 in Table 1). This difference was proposed to be caused by the higher MMR efficiency in the lagging strand that is linked to the ready availability of initiation sites provided by Okazaki fragment termini (Nick McElhinny et al., 2010a). The loss of *RNH201* (the catalytic subunit of RNase H2 in yeast) led to an ~1.5-fold increase in mutation rates in both leading and lagging strands in this system (Clark et al., 2011; Kim et al., 2011; Nick McElhinny et al., 2010b). We now show that the *rnh201Δ* mutation failed to further increase the mutation rate in the MMR-deficient background (*rnh201Δ msh2Δ* in Table 1). Moreover, the relative contribution of RNase H2 toward replication fidelity was higher on the leading strand (24% of the total MMR activity) and amounted to twice that seen on the lagging strand (10% of the total MMR activity). In addition, DNA sequencing revealed that 100% of the mutants displayed C-to-A transversions in the leading strand, the signature phenotype of a MMR defect in this reporter system (Pavlov et al., 2003). Taken together, the data indicate that the increased mutagenesis detected in the *rnh201Δ* mutant is likely linked to diminished MMR efficiency.

A key hallmark of MMR deficiency is instability of the so-called microsatellites—sequence elements consisting of repeats of mono-, di-, or trinucleotides. These motifs are prone to strand misalignments during replication, which give rise to insertion/deletion loops (IDLs). Because IDLs are repaired predominantly by MMR, cells lacking this repair pathway display microsatellite instability (MSI), which can be measured in suitable reporter assays (Strand et al., 1993). We decided to make use of this phenomenon to determine the effect of RNase H2 deficiency on MMR efficiency. In our assay, a T_9 repeat was introduced in-frame into the *URA3* coding sequence, and the construct was integrated into the *ADE2* locus in the *S. cerevisiae* genome. Alterations other than ±3 nucleotides in the length of the T_9 repeat inactivate the *URA3* gene and render the cells resistant to 5-fluoroorotic acid (FOA). As anticipated, the T_9URA3 marker was highly unstable in the MMR-deficient *msh2* or *mlh1* strains; we observed a 64- and 52-fold increase in mutation rates relative to WT, respectively (Table 2). Importantly, in the absence of *RNH201*, we observed an overall 4-fold increase in mutation rates relative to WT, whereas no significant increase was observed when *RNH201* was deleted in the MMR-deficient background. Sequencing of a 242 bp region of *URA3* flanking the T_9 repeat in 50 FOA-resistant *rnh201Δ* clones revealed an 8.5-fold increase in the rate of −2 deletions compared to WT, which are most likely due to mutagenic processing of ribonucleotides in DNA catalyzed by topoisomerase I (Kim et al., 2011). Importantly, we also detected a 7.4-fold increase in the rate of −1 deletions in the T_9 repeat of the *rnh201Δ* strain compared to WT. This is a key hallmark of MMR deficiency, as clearly substantiated by the finding that all mutations identified in the *msh2Δ* and *rnh201Δmsh2Δ* strains were −1 deletions in this motif. Taken together, the above results show that the eukaryotic MMR system can and does use intermediates of processing of misincorporated ribonucleotides as additional repair initiation sites.

DISCUSSION

How organisms lacking MutH homologs direct MMR to the continuous leading stand has puzzled researchers for decades. The requirement for the endonucleolytic activity of MutL or its eukaryotic orthologs (Kadyrov et al., 2006) showed that this process was dependent on the introduction of discontinuities where EXO1 can load, but it was only with the characterization of an association between MutLα and PCNA (Pluciennik et al., 2010) that the molecular mechanism of the process could be understood (Peña-Diaz and Jiricny, 2010). Given the strong mutator phenotype of cells lacking the MutLα endonuclease, it must be assumed that this enzymatic activity represents the key determinant of strand directionality in MMR, similarly to MutH in *E. coli* (Zell and Fritz, 1987). However, DNA polymerases incorporate several noncanonical nucleotides into nascent DNA, such as deoxyuridine (Andersen et al., 2005), 8-oxo-deoxyguanosine (Maki and Sekiguchi, 1992), and ribonucleotides (Nick McElhinny et al., 2010b; Reijns et al., 2012). Because metabolism of these nucleotides involves excision, we wondered whether the strand breaks generated during their removal might be deployed by the MMR system for initiation of excision in cases where a misincorporated nucleotide is in the vicinity. We decided to investigate the link between MMR and the processing of ribonucleotides, because their abundance in nascent DNA is high (Reijns et al., 2012)—particularly in the leading strand, due to the fact

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Table 2. Mutation Rates within the T_9URA3 Cassette in the Indicated *S. cerevisiae* Strains

Genotype	Overall Mutation Rate × 10^{-7}	Fold Increase Relative to WT	−1 Deletion Rate at the T_9 Repeat × 10^{-7}	Fold Increase Relative to WT
Wild-type	0.24 (0.17 – 0.36)[a]	1	0.015 (3/45)[b]	1
mh201Δ	1.02 (0.79 – 1.55)[a]	4.3	0.12 (6/50)[b]	7.3
msh2Δ	15.4 (11.77 – 19.62)[a]	64.2	15.4 (31/31)[b]	917
msh2Δmh201Δ	15.21 (13 – 19.07)[a]	63.4	15.21 (28/28)[b]	905
mlh1Δ	12.57 (10.04 –19.92)[a]	52.4	ND	ND
mlh1Δmh201Δ	13.53 (10.33 – 17.31)[a]	56.4	ND	ND

The data were obtained from 32-50 independent cultures of each strain. Fold increases were calculated relative to the wild type strain. Mutation rates and confidence levels were calculated by the method of Lea and Coulson (Lea and Coulson, 1949). The −1 deletion rates at the T_9 repeat were calculated from the formula [del rate = (nr. of sequenced mutants with −1 deletions in T_9/total number of sequenced mutants) x overall mutation rate]. ND, not determined.

[a]Numbers in parentheses represent 95% confidence levels.

[b]Numbers in parentheses represent the number of colonies with −1 deletions versus the number of sequenced colonies (see the Experimental Procedures).

that they are inefficiently excised by the proofreading activity of pol ε (Williams et al., 2012). Moreover, the loss of RNase H1/2 gives rise to higher mutation rates and genomic instability (Clark et al., 2011; Lazzaro et al., 2012; Reijns et al., 2012), and, although it was proposed that the mutagenicity of ribonucleotides might be caused by their misincorporation opposite a noncomplementary template nucleotide (Clark et al., 2011; Shen et al., 2012), this possibility appeared to us unlikely, given that the bases of ribonucleotides form perfect hydrogen bonds with DNA bases. We now show that the presence of ribonucleotides in nascent DNA and their processing by RNase H2 increases MMR efficiency, most likely through providing the system with additional strand breaks that can be utilized as entry sites for EXO1 (Figure 4). The contribution of this pathway to MMR fidelity is small, because it requires that the ribonucleotide and the mispair are within less than ~1 kb of each other. However, if the MMR system also makes use of intermediates of processing of other nascent strand markers such as deoxyuridine and 8-oxo-deoxyguanosine, then this mechanism may play a substantially more important part in the initiation of MMR than first thought.

Given that the presence of ribonucleotides in DNA is linked to genomic instability (Clark et al., 2011; Lazzaro et al., 2012; Reijns et al., 2012), it might appear surprising that replicative polymerases have not evolved to exclude them. Our findings suggest that the presence of rNMPs, possibly together with other markers of nascent DNA, may have been tolerated during evolution (Andersen et al., 2005; Russo et al., 2004), because they aid metabolic processes that need to distinguish between the parental and daughter DNA strands.

EXPERIMENTAL PROCEDURES

Substrates, Nuclear Extracts, and MMR Assays

The substrates were generated as described previously (Baerenfaller et al., 2006). Briefly, the hetero- and homoduplexes were constructed by primer extension using the oligonucleotides listed below as primers and the single-stranded phagemid DNA as template. Depending on the orientation of the ribonucleotide and the nick, two different ssDNA templates were used (pRichi-350topSalI creates 3′ substrates, and pRichi-2850topSalI creates 5′ substrates). After primer extension, ligation, and isolation of the desired supercoiled heteroduplex substrates on CsCl gradients, MMR assays were carried out as described (Baerenfaller et al., 2006).

Unless otherwise specified, the MMR reactions were carried out with 100 ng DNA substrate and 100 μg nuclear extracts in a total volume of 25 μl in a buffer containing 20 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 110 mM KCl, 1 mM glutathione, 50 μg/ml BSA, 100 μM dNTPs, and, where indicated, 2 μCi of [α-^{32}P] dCTP. The reactions were incubated at 37°C for 30 min. For time course experiments, 8 μl aliquots were withdrawn at the indicated time points. The reaction was stopped by adding an equal volume of 2× stop solution containing 1 mM EDTA, 3% SDS, and 5 mg/ml Proteinase K. The samples were incubated at 45°C for 30 min, purified on Mini-Clean columns (QIAGEN), and subjected to restriction digests. The digested DNA was resolved on 1% agarose gels. Nuclear extracts used in this study were prepared from HEK293, LoVo, 293TLα , and mouse embryonal fibroblasts obtained from RNase H2, +/−, −/− mice as indicated in the figures.

Primers

All primers were obtained from Microsynth (Balgach, Switzerland). The sequences are indicated below. The SalI restriction site (GTCGAC) is underlined. The mispaired residue is highlighted in bold. The position of the ribocytidine is indicated in lower case.

T/G-SalI primer is as follows: 5′-CCAGACGTCTGTTGACGTTGGGAAGCTTGAG-3′. 5′ rC-T/G primer is as follows: 5′-GAATTGTAATAcGAACACTATAGGGCGAATTGGCGGCCGCGATCTGATCAGATCCAGACGTCTGTTGACGTTGGGAAGCTTGAG-3′. 5′-rC-C/G primer is as follows: 5′GAATTGTAATAcGAACACTATAGG-3′. 3′ rC-T/G-60 primer is as follows: 5′-CCAGACGTCTGTTGACGTTGGGAAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTcATAGCTGTTTCCTGTGTG-3′. 3′ rC-C/G primer is as follows: 5′-GCGTAATCATGGTCATAGCTGTTTCC-3′. 3′ rC-T/G-308 primer is as follows: 5′-GCTTCCTCGCTCACTGAGTCGCTGcGCTCGGTCGTTC-3′.

Antibodies and Recombinant Proteins

The RNASEH2A antibody for western blots (rabbit polyclonal, GeneTex) was used at a dilution of 1:1,000, MSH2 (mouse monoclonal, BD Transduction Laboratories) was used at a dilution of 1:1,000, and MLH1 (mouse monoclonal, Oncogene) was used at a dilution of 1:1,000. The RNase H2 (sheep polyclonal) antibody used for immunodepletion was generated by A.P.J. Recombinant MutSα and MutLα were expressed and purified in our laboratory. RNase HI and HII were obtained from New England BioLabs.

Band-Shift Assay

This was performed essentially as described (Cejka et al., 2005). The oligonucleotide heteroduplexes were created by annealing 5′ radiolabelled (*)

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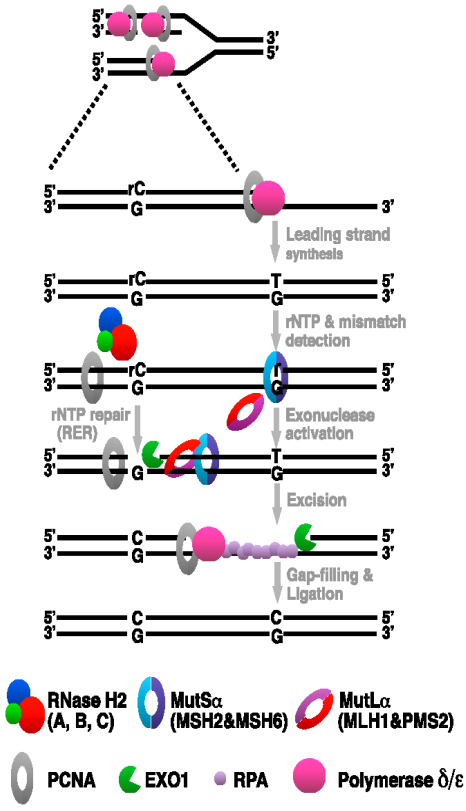

Figure 4. Putative Mechanism of Ribonucleotide-Mediated Strand Discrimination during Eukaryotic MMR

Replicative polymerases such as polymerase epsilon (pol ε) erroneously incorporate single ribonucleotides (1 rNMP per 1,250 dNMPs) into the nascent DNA strand. RNase H2 cleaves the DNA 5′ from the rNMP. In the event that the polymerase generates a mispair in the vicinity, the mismatch-activated MMR proteins can "hijack" the transient RNase H2-mediated nick as a loading site for EXO1, which then degrades the error-containing strand.

oligonucleotide 5′CTCAAGCTTCCCAACGTC**G**ACAGACGTCTGG3′ with the following unlabeled oligonucleotides: T/G mismatch, 5′-CCAGACGTCTGT**T**G ACGTTGGGAAGCTTGAG-3′; C/G match, 5′-CCAGACGTCTGT**C**GACGTT GGGAAGCTTGAG-3′; and rC/G mismatch, 5′- CCAGACGTCTGT**c**GA CGTTGGGAAGCTTGAG 3′, where boldface nucleotides define the residues creating the mismatches. The position of the ribocytidine is indicated in lowercase. The binding reaction mixtures contained 40 fmol oligonucleotide duplex T/G*, C/G*, or rC/G* and MSH2/MSH6 (100 or 250 ng). Protein-bound substrates were separated from free probes by electrophoresis on a 6% native polyacrylamide gel eluted with TAE.

In Vitro Nicking Assay

Supercoiled DNA substrates (100 ng) were incubated with 1 U of *Nt.Bst*NBI, RNase HI, or RNase HII in buffers recommended by the manufacturers. Nicking assays with recombinant human RNase H2 (WT or the catalytically inactive DD/AA mutant, 0.1, 1, and 10 μM) were carried out with 100 ng of supercoiled rC-T/G substrate in a buffer containing 20 mM Tris.HCl (pH 7.6), 5 mM $MgCl_2$, 110 mM KCl, 1 mM glutathione, and 50 μg/ml BSA. The substrates were incubated at 37°C for 45 min. The products were then loaded on a 1% agarose gel and visualized with GelRed.

Mismatch-Dependent Strand Degradation Assays

The experiments were performed essentially as described (Genschel and Modrich, 2003) with some modifications. Briefly, 100 ng of the homo-/heteroduplex was treated with 1 U of bacterial RNase HII or 10 μmole human RNase H2 (WT or DD/AA) in a reaction containing the recombinant MMR proteins (150 ng MutSα, 100 ng MutLα, 70 ng RPA, and 1.6 ng of EXO1 in 20 mM Tris.HCl [pH 7.6], 1 mM glutathione, 5 mM $MgCl_2$, 0.05 mg/ml BSA, 3 mM ATP, 100 mM KCl). Mismatch-provoked excision reactions were allowed to proceed for 7 min at 37°C, following which the samples were digested with HindIII and XmnI at 37°C overnight and analyzed on 1% agarose gels containing GelRed.

siRNA and Knockdown Experiments

293 cells were transfected with 40 pmol of siRNA against luciferase (siLuc), RNase H1, RNase H2a, or both H1 and H2a, using RNaiMax (Invitrogen) as per the manufacturer's protocol. For each knockdown, six 15 cm dishes were transfected. The following day, the plates were trypsinized and scaled up into 20 15 cm dishes. Forty-eight hours posttransfection, the nuclear extracts were prepared as described above. The siRNAs used in this study were as follows: siLuc, 5′-CGUACGCGGAAUACUUCGA-3′; siRNase H1, 5′-GAAGACAAGUGCAGGGAAA-3′; and siRNase H2A, 5′-GGACUUGGAUA CUGAUUAU-3′.

RNase H2-Immunodepletion of Nuclear Extracts

Protein A/G beads (SantaCruz) were washed twice with binding buffer (30 mM HEPES.KOH [pH 7.5], 7 mM $MgCl_2$) and incubated with the RNase H2 antibody at 4°C for 3 hr (10 μl of the serum was used to bind 25 μl of the bead slurry). They were then washed thrice with the binding buffer and subsequently used to immunodeplete the nuclear extracts. For 150 μg of nuclear extracts, 10 μl of antibody-preadsorbed beads were used. Mock-depleted nuclear extracts were obtained by incubating with the beads alone. The immunodepletion was carried out for 30 min at 4°C, and the MMR assays were performed immediately.

In Vivo Mutagenesis Assays

Strand-specific mutation rates in *S. cerevisiae ogg1Δ* strains were measured using the *ura3-29* marker as described previously (Pavlov et al., 2003). The *RNH201* and *MSH2* deletions were generated by standard genetic procedures. Mutation rates were obtained by fluctuation tests using 9–20 independent cultures. The 95% confidence limits for the median and the differences between mutation frequencies using the Mann-Whitney nonparametric criterion were determined as described (Dixon and Massey, 1983).

In the second assay, we used a reporter cassette containing a $(T)_8$ repeat within the *URA3* open reading frame that had been introduced into the *ADE2* locus by standard genetic techniques. Mutation rates were calculated from fluctuation analysis with 32–50 independent cultures using the Lea-Coulson method of median (Lea and Coulson, 1949), based on the appearance of mutants resistant to 5-FOA. The fluctuation analysis calculator (FALCOR) software was used to calculate the mutation rate and confidence levels (Hall et al., 2009). The strains were derivatives of FF18733/FF18734 (Cejka et al., 2005).

DNA Sequencing

Genomic DNA from ten URA+ clones per *S. cerevisiae ogg1Δ* strain was purified using an YDER Kit (http://www.piercenet.com/browse.cfm?fldID=06010499). The URA3 region containing the ura3-29 mutation was amplified by PCR using the primers 5′-GAACGTGCTGCTACTCATCC-3′ and 5′-CATTCTGCTATTCTGTATAC-3′. The PCR product was sequenced by Cogentech (http://www.cogentech.it/) using the primer 5′-TAGTTGAAGCATT AGGTCCC-3′.

Mutations flanking the T_8 repeat were identified by sequencing a 500 bp fragment covering the first 242 bp of the URA3 coding sequence (including

Please cite this article in press as: Ghodgaonkar et al., Ribonucleotides Misincorporated into DNA Act as Strand-Discrimination Signals in Eukaryotic Mismatch Repair, Molecular Cell (2013), http://dx.doi.org/10.1016/j.molcel.2013.03.019

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the T_9 repeat), amplified by PCR using primers 5′-GCATTGGATGGTGG TAACG-3′ and 5′-GGAACGACAGTACCCTCATAAC-3′. PCRs were carried out using ReddyMix PCR Master Mix (Thermo Scientific) and 10 ng of yeast genomic DNA (95°C 5 min; then three cycles of 94°C 30 s, 65°C 30 s, and 72°C 45 s; then three cycles of 94°C 30 s, 62°C 30 s, and 72°C 45 s; then three cycles of 94°C 30 s, 59°C 30 s, and 72°C 45 s; then 35 cycles of 94°C 30 s, 56°C 30 s, and 72°C 45 s; then 72°C for 10 min). Purified PCR amplification products from 28–50 independent clones were sequenced using dye-terminator chemistry and electrophoresed on an ABI 3730 capillary sequencer (Applied Biosystems). Sequencing data were analyzed using Sequencher 4.8 (Gene Codes).

ACKNOWLEDGMENTS

The authors thank Kalpana Surendranath and Anja Saxer for recombinant MutSα and MutLα, Murat Aykut for sharing reagents for the yeast work, Ailsa Revuelta for technical support, Stefano Ferrari for recombinant EXO1, Pavel Janscak for recombinant RPA, Thomas A. Kunkel for yeast strains, and Luca Gianfranceschi for help with statistical analyses. The work was supported by Swiss National Science Foundation grants 310030A-118158 and 310030B-133123, and by European Community's Seventh Framework Programme (FP7/2007-2013) grant agreement number HEALTH-F4-2008-223545 to J.J.; by grants from AIRC and MIUR to M.M.-F.; and from MIUR (FIRB RBFR10S3UQ) to F.L. The financial support of Telethon-Italy (grant number GGP11003) is also gratefully acknowledged. A.P.J. and M.A.R. were supported by a MRC Senior Clinical Fellowship. J.J., M.M.-F., M.M.G., P.C., and P.P. conceived the study and devised the experiments; M.M.G. designed and M.A.-B. constructed all the substrates generated in this study. M.M.G. carried out the biochemical experiments; F.L. and M.M.-F. carried out the strand-specific genetic studies; M.M.G., with the help of M.O.-P. and P.C., carried out the T_9URA3 genetic studies. M.O.-P. performed the band-shift assay and assisted in the preparation of nuclear extracts and substrates. M.A.R. and A.P.J. carried out the sequencing of the T_9 repeats and provided the RNase H2 polyclonal antibody and the RNase H2 knockout MEFs; J.J., M.M.-F., and M.M.G. wrote the manuscript.

Received: October 23, 2012
Revised: January 30, 2013
Accepted: March 14, 2013
Published: April 18, 2013

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Molecular Cell

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6 Discussion

6.1 Studying O6-methylguanine adducts in *in vitro* replicating systems

Methylating agents such as MNU, MNNG and their clinical counterpart temozolomide generate a broad spectrum of DNA damage in cells and sensitize them to killing, both alone and in combination with other damaging agents, a characteristic which is of use in the clinic for the treatment of melanoma, certain leukemias and glioblastoma. The finding that one lesion in this particular context, O6-methylguanine (^{me}G), is accountable for most of the cytotoxic action of these agents has paved the way for a series of follow-up observations that should serve to increase the usage specificity of these agents. Given that, under physiological conditions, ^{me}G can be directly repaired by MGMT and that only when MGMT is inhibited does MMR take over and sensitize cells by a factor of 100 when compared to controls, the study of the exact contribution and nature of MMR recognition of this mutagenic, cytotoxic and carcinogenic lesion is of critical importance.

Previous *in vitro* and *in vivo* studies have painted the following picture:

- Treatment with S_N1-type alkylators induces a G2/M arrest in the second phase of the cell cycle.
- ^{me}G is the main cytotoxic lesion; it is in the template during replication.
- This phenomenon is dependent on MMR proficiency in cells. Due to the strand specificity of MMR, ^{me}G is irreparable and attempted MMR will restore rather than repair the original mismatches.
- Primer extension assays with recombinant replicative polymerases have shown that either C or T can be misincorporated opposite ^{me}G.
- The MMR machinery includes the replicative polymerase pol δ. Also reported to be involved are error-prone TLS polymerases such as pol κ and pol η.
- The second S phase post-treatment is accompanied by strong checkpoint activation mediated mainly by the ATR kinase.
- Interactions between MMR proteins and checkpoint proteins such as ATR and Chk1 have been reported.

Two non-mutually exclusive models partake in the discussion of this MMR-induced cytotoxicity. The direct signaling model posits that recognition of the ^{me}G mismatch is

sufficient to trigger cell death, since MMR can communicate directly with the ATR checkpoint machinery. The futile cycling hypothesis does not exclude this interaction but, based on the G2/M arrest ocurring after the second S phase, hypothesizes that it is rather the well-intended albeit futile processing of the mismatches by MMR which ultimately gives rise to structures that hinder replication, structures which would form behind the replication fork during the first replication but ahead of the second. Reports on both scenarios are divided.

We established the *Xenopus* egg extract technique system in the laboratory, because it allows the single, naturally-synchronous replication of damaged templates in a contained *in vitro* setting where manipulation is more feasable. Other studies with biochemical emphasis worked with recombinant proteins and nuclear extracts where one can monitor MMR activity on a single ^{me}G, but never in the context of replication. The main questions we had pertained to the ^{me}G lesion itself: does MMR recognize and bind to it throughout replication? is there an accumulation of ^{me}G/T mismatches and does their presence elicit a checkpoint response? Can a single ^{me}G generate structures in DNA that could inhibit a second round of replication?

An important achievement was the generation of *Xenopus* antibodies that recognize the MMR proteins in the extract. Then followed the analysis of MMR activity in response to methylation damage in the extract.

In our study we could observe dynamic recruitment of main MMR players such as Msh2, Msh6, Mlh1 and Exo1 to the chromatin during replication in response to treatment with the S_N1-type alkylators MNNG and MNU. This was distinct from normal recruitment to the chromatin during replication, in that the recruited proteins remained in the chromatin after replication was completed and also because it was ^{me}G-dependent. All 4 proteins could be detected this way, indicating that MMR proteins continue their attempts to process the ^{me}G lesion behind the replication fork.

We next assessed what previous studies had implied. Namely, that ^{me}G recognition by MMR is accompanied by recruitment of a non-canonical ATR checkpoint machinery, where recruitment of proteins such as Rad17 or the 9-1-1 complex is dispensable, but where at least recruitment of ATR and its interaction partner ATRIP, as well as its activator TopBP1, should be observed. In replication reactions where increasing doses of the methylating agent were used, we were clearly able to detect a dose- and ^{me}G-

dependent recruitment of MMR proteins to the chromatin, but an ATR machinery co-dependency was not observed. Treatment with MMS, which elicits cell death through the generation of DSBs and is considered a radiomimetic but also generates a small amount of ^{me}Gs, led to strong checkpoint machinery recruitment. This served to underscore that presence of ^{me}Gs is not warranted for such a recruitment.

We were also able to confirm that since S_N1-type alkylators generate a plethora of different lesions, increasing the treatment dose can indeed activate a checkpoint but that this is MMR-independent and is most likely due to the processing of other lesions in close proximity to one another.

After confirming this ^{me}G-dependent activity we switched to a different extract system, which allowed the replication of specific single ^{me}G-containing substrates as well as of control substrates. *Xenopus* mismatch repair assays had confirmed that ^{me}G/T was a better substrate for MMR than ^{me}G/C, so we were intrigued by the possibility that presence of either a ^{me}G/C or ^{me}G/T could trigger a response in the extract that could inhibit replication. This would mimic a scenario where the only difference between the first and second S phase was the initial presence of ^{me}G/C immediately after treatment as opposed to the presence of ^{me}G/T in the DNA generated during the previous S phase. This distinction can only be made in a replication extract with specific ^{me}G substrates and not with MNNG or MNU treatment as is done in cell culture work.

Replication of all substrates was comparable. As observed by [α-^{32}P]dCMP incorporation into the substrate during replication, only a slight decrease in product formation could be detected. When we analyzed the kinetics of checkpoint activation during this replication, we could detect, similarly to the experiments with the MNU or MNNG-pretreated chromatin, that there is no checkpoint activation at the end of replication. We could detect, however, a transient checkpoint activation in response to lesion recognition. This was interesting, since it confirms observations made by others about MMR directly interacting with the ATR checkpoint machinery to signal the presence of a mismatch. The fact that it is short-lived and doesn't affect replication makes it hard to imagine that cell arrest or cell death could result from it. Also, we observed that incubation of a T/G substrate could also lead to a similar transient checkpoint activation as can be seen in Fig. 18. This result is preliminary and puzzling. Since T/Gs are more common physiological mismatches that can be repaired, it would indicate a general MMR

recognition mechanism that is not ^{me}G specific. Whether this is due to knicking of the DNA upon mismatch recognition or whether it causes a change in the chromatin environment recognized by proteins that monitor replication remains to be established. Recently, a report by Noonan et al. reported a related observation in cells [324]. When they analyzed the cell cycle profiles and checkpoint response to MNNG in TK6 cells (MMR proficient and MGMT deficient) and compared it to MMR deficient TK6/MMR$^-$ as well as MGMT proficient TK6/MGMT$^+$ cells, they also observed weak checkpoint activation (ATR, Chk1, γH2AX) and transient replication slow-down during the first S phase. They were quick to acknowledge, however, that this damage signaling occuring upon initial lesion recognition is not sufficient to invoke cell death and that apoptosis can be observed only after cells progress into the second round of replication, where futile cycling presumably leads to replication fork collapse. What then is the difference between the first and second rounds of replication that allows survival during the first cell cycle but not during the second?

Work done by Mojas et al. had previously reported that treatment of cells with MNNG could leave small gaps behind the replication fork. It was discussed that these could be the main culprit in the observed cytotoxicity. Small gaps behind the fork that are being repaired could probably escape checkpoint monitoring since MMR iterative repair attempts behind the fork would not allow prolongued exposure of ssDNA regions (RPA-coated ssDNA regions being the assumed substrate for ATR activation). During the second S phase, however, the replication fork would encounter them and collide with MMR. It remains unclear whether MMR could recognize these lesions ahead of the fork and continue its repair attempts. It has been shown that MMR activity can interfere with chromatin assembly to access mismatches, so it remains a possibility [100, 103]. In any case, unwinding of the ^{me}G-containing gapped chromatin alone would lead to replication stress.

Final proof that MMR activity on ^{me}G lesions leads to gaps was still missing, because the experimental setup in cells is not available and in non-replicating extracts distinguishing normal MMR activity from futile cycling is difficult, since both repair of a T/G mismatch and a ^{me}G-containing mismatch would incorporate new nucleotides in the excised strand and be detected by the use of radioactivity. In *Xenopus* egg extracts, gapped substrates have been shown to trigger a strong checkpoint response, so we asked whether

processing of ^{me}Gs could indeed lead to gap formation after replication is complete. The result obtained from the replication of a ^{me}G- containing substrate in the *Xenopus* extract was clear: formation of new mismatches during replication leads to lesion recognition and persistent MMR activity on the ^{me}G. Isolation of the replicated substrate and a radioactive fill-in with Klenow polymerase revealed the presence of gaps opposite the ^{me}G which, as expected, was still present in the template strand after replication. With this final observation we were able to confirm MMR futile cycling in reponse to ^{me}G. Even though it is known that gapped substrates lead to checkpoint activation in the extracts, we wanted to confirm this during a re-replication reaction in a more physiological scenario with MNU-damaged sperm chromatin. This final experiment confirmed that, after replication of a ^{me}G-containing template is completed and the template is isolated, re-replication of the damaged template is severely impaired in the extract where MMR has taken over processing of the ^{me}G lesions and is creating gaps opposite it.

This analysis has provided us with biochemical evidence supporting that abortive repair of ^{me}G lesions can extend to the formation of gapped DNA. Nevertheless, the direct consequence of the presence of these gapped intermediates remains open to discussion. That DSBs could arise upon replication forks encountering the lesions is evident. Whether other mechanisms, such as fork reversal, which can also activate the DNA damage response and which is emerging as a common ocurrence during replicative stress [325-327], could also play a role in activating the DNA damage response parallel or prior to this remains to be analyzed and would be interesting when thinking of possible combinational therapy.

Studies carried out to date have assumed that whatever transpired in the DNA during the first S phase, such as the formation of gaps, persists during mitosis, probably due to the condensed nature of the chromatin and relative inaccessability of repair proteins to damaged DNA. Even though MutSα is expressed in G2/M, whether its activity is tightly regulated by post-translational modifications that inhibit it during this time is unknown. In general, the DNA damage response in mitotic cells remains poorly understood and MMR processing of the lesion in mitosis seems unlikely. What is known, is that deleterious lesions such as DSBs induced by IR do not hinder mitotic progression per se, but they do lead to H2AX phosphorylation, for example, thus priming the cell for a full-blown DNA damage response. Similarly, recognition of the ^{me}Gs during the following G1 phase by

non-canonical MMR [310, 328] should not be ruled out but is also beyond the scope of this thesis.

Finally, the generation of antibodies specific for *Xenopus* MMR proteins provides a useful tool in studies related to their role in replication or in the repair of other lesions, such as mispairs generated upon oxidative damage or ICLs. There are preliminary indications that the affinity-purified antibodies can also be used for immunoprecipitation, which would be of interest for possible protein-protein interaction studies in the XEE. Additionally, as seen in Fig. 19 of this thesis, it is also possible to replicate biotinylated substrates in the HSS+NPE extract, which provides the unique opportunity of chipping the susbtrate via streptavidin-bead pulldown and detecting proteins bound specifically to the ^{me}G either by western blotting or mass-spectrometry. In this experimental setup, the *Xenopus* MMR antibodies could be used to detect MMR proteins binding to ^{me}G as a positive control.

6.2 Recognition of O6-methylguanine adducts by MutSβ and its possible implications

Perhaps the most solid evidence in support of the direct signaling hypothesis is the report of separation of function mutants in mouse Msh2 and Msh6 that allow for nucleotide binding but not further lesion processing, and which still activate apoptosis in response to treatment with DNA damaging agents such as cisplatin and MNNG. In the case of MNNG at least, this response involves a G2/M arrest in the second cell cycle after treatment. This is where this model begins to contradict itself, since lesion recognition occurs during a first S phase and this hypothesis does not allow for an explanation as to why arrest and cell death occur after a second S phase. In strong opposition to reports supporting the direct signaling hypothesis, others have reported a definite requirement for downstream MMR players such as MLH1 and EXO1 in this apoptotic response, both of which are involved in the excision step during MMR [206, 329]. Also noteworthy, a human cell line harboring a mutation in MSH6 that disables MutSα sliding along the DNA and is considered MMR deficient like the mutant MEFs, is resistant to MNNG [330, 331].

We hypothesized that the disparity between these studies could maybe be explained by recognition and processing of the ^{me}G lesion by a MMR player which has been reported to have partial redundancy with MutSα: MutSβ. Neither of the aforementioned studies established whether MutSβ was still active in the mouse embryonic fibroblast (MEF) extract they tested for MMR activity. In fact, the repair deficiency of these extracts was observed by using mismatches other than the most relevant ^{me}G-containing mismatches. Moreover, it is interesting to note that sensitivity to one of the chemotherapeutic agents which was used in the studies, cisplatin, could be MutSβ–related, since it has been implicated in this type of repair. This was not discussed because, at the time, a role for MutSβ in ICL repair was considered unlikely. This heterodimer, composed of MSH2 and MSH3, was also recently reported to be involved in the cytotoxic response to 5-fluorouracil [188], a novel observation that implicated it in recognition of a lesion traditionally assigned to MutSα and BER [187].

Xenopus MMR assays first indicated that immunodepletion of Msh6 from the extract could abolish repair activity for all but the ^{me}G/T substrate. The fact that there was residual Msh2 in the extract prompted us to investigate further whether Msh2 dimerised

with Msh3 could still be attempting ^{me}G repair. Preliminary *in vitro* experiments conducted so far would indicate that this is indeed the case. Recombinant human MutSβ showed some affinity for ^{me}G-containing duplexes in an EMSA, and MMR-deficient LoVo extracts supplemented with either MutSα or MutSβ restored repair activity on a ^{me}G/T substrate. We were particularly interested in mismatch recognition in the HCT15 cell line, which only expresses MutSβ. Surprisingly, it not only displayed repair activity on a ^{me}G/T susbtrate, but also on a T/G substrate which contradicts the results obtained by a different laboratory [293]. Here, a MMR assay performed with HCT15 cell extract showed no repair activity for T/G (^{me}G/T recognition was not tested).

We cannot yet assess whether MutSβ is able to recognize this lesion *in vivo* or whether this recognition would lead to canonical MMR machinery activation and recruitment. Reports of MutSβ involvement in ICL repair have been quick to point out that it occurs independently of MLH1 recruitment, for example [202]. Nevertheless, the possibility of MutSβ recognition of ^{me}G lesions remains intriguing.

Our first attempt at analyzing this further has so far brought inconclusive results. We monitored recruitment of MMR proteins to chromatin after a 3 h treatment with MNU in U2OS cells but, at least in this cell line, MSH3 is strongly present on chromatin regardless of treatment. Whether this can be attributed to the traditional role of MSH3 in IDL repair, or to a possible role in the repair of other types of endogenous damage such as DSBs is a possibility, since MSH3 deficiency, similarly to a BRCA1 deficiency which impairs DSB repair, has been reported to further sensitize cells to PARP inhibition [332].

A previous study conducted in the HCT15 cell line compared its survival to MNU treatment with that of the MSH6-proficient counterpart HCT15+chromosome 2 (HCT15+Chr2) cell line [323]. This study showed only a slight difference in sensitivity towards MNU, not obvious resistance as is expected from a MMR deficient cell line. Since this cell line only expresses MutSβ, it would be interesting to investigate whether it is more resistant to treatment with MNU upon downregulation of MSH2 or MSH3 and perhaps even show a more obvious recruitment to ^{me}G lesions. We find that this would be more conclusive than compairing different cells lines (HCT15 and HCT15+Chr2), since western blot analysis of the MMR components in the cell extracts revealed that the amount of MSH2 differs significantly in these two cell lines and this could be a factor affecting sensitivity to treatments.

It would be interesting to test whether this can be seen in other cell lines paired against each other, such as the human promyelocytic cell lines HL60 and HL60R, the former of which overexpress MSH3 and is considered MMR deficient [333].

With regards to the point mutations in Msh2 or Msh6 that might uncouple damage signaling from repair, it would be important to see whether the said mutations also affect MutSβ translocation along the DNA, as it was observed for MutSα. This cannot be ruled out because structural studies have shown that these two heterodimers have distinct binding modes for their substrates and MutSβ appears to be more flexible when it comes to lesion recognition and binding [334, 335]. That different residues in both heterodimers are responsible for lesion recognition or processing can be exemplified by the fact that the conserved Phe-X-Glu motif that both the prokaryotic MutS homodimer and MutSα possess for stacking onto the mispair base is missing in both yeast and human MSH3. Instead, there is a lysine instead of phenylalanine and lysine or arginine instead of glutamate [336].

Further *in vitro* and *in vivo* studies employing a MutSβ carrying the mutation in MSH2 would be neccessary to bring clarity to the discussion about ^{me}G recognition and its consequences. In general, not enough is known about how MutSβ and MutSα activities are regulated during S phase and how their respective interactions with other proteins help dictate their cellular function.

7 Conclusions and perspectives

The MMR machinery is a highly-conserved DNA repair mechanism that can be found in bacteria, plants and humans. This pathway increases the fidelity of replication by several orders of magnitude. It also tackles mispairs arising from treatment of cells with chemotherapeutic agents such as temozolomide. In this context, MMR activity sensitizes cells to killing by these agents at lower doses than would be neccessary for non-MMR mediated killing. In a clinical context, this means less harmful side-effects for patients treated with these agents.

The importance of this pathway and its repair activity for maintenance of genomic integrity becomes evident when mutations in components such as MSH2 and MLH1 are correlated with susceptibility to human non-polyposis colorectal cancer (HNPCC).

This study focused on the MMR-dependent mode of action that significantly attenuates tolerance to treatment with S_N1-type alkylators such as MNNG, MNU or temozolomide. In this, more focus on the interplay between MMR, signaling activity and mismatch recognition during replication was of vital importance, to be able to correctly assess and/or develop strategies for more specificity of these treaments. This was possible with the use of the XEE technique and the generation of specific tools for the study of *Xenopus* MMR, which is closely related to human MMR. Parallel to this, a possible role for the MutSβ in ^{me}G processing which merits further investigation was uncovered.

At a time where screening of patients for specific mutations is becoming increasingly common and neccessary for treatment discussion (e.g. BRCA1 and BRCA2 mutations in breast and ovarian cancer), where sufficient knowledge is available that one can envision the direct consequence of some defective DNA repair genes or DNA repair protein function, and where targeted delivery of DNA damage to cancerous cells is a priority, the pursuit of knowledge in the DNA repair field that has clinical application is vital to the scientific community, but should be conducted with care. Additional thorough investigation of the MMR pathway, its interactome, its post-translational regulation and its cell-cycle regulation are areas of interest that would help amass and apply knowledge in this field.

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9 Acknowledgements

This is a refrain of a Nat King Cole song which, like many of the people mentioned in my words of thanks, accompanies me whenever a sturdy experiment didn't work as you expected it to and you just need to keep going:

Smile though your heart is aching
Smile even though it's breaking
When there are clouds in the sky, you'll get by
If you smile through your fear and sorrow
Smile and maybe tomorrow
You'll see the sun come shining through for you

First and foremost I would like to thank my PhD supervisor Prof. Josef Jiricny with heartfelt sincerity. I am extremely grateful for the constructive and encouraging learning environment which he provided during these past few years and believe that it has allowed me to understand and conduct scientific endeavours as well as provided me with fond memories of the laboratory and the IMCR. I couldn't have picked a better boss for carrying out my PhD.

I would also like to thank my committee members for their supportive guidance throughout my degree. Prof. Ulrike Kutay has been instrumental in my academic development and has challenged my understanding of my PhD project. Dr. Vincenzo Costanzo has always been very enthusiastic about my work and he has continuously provided a supportive voice for the project and even invited me to stay in his lab to learn more about the *Xenopus* egg extract technique.

I owe particular thanks to all past and present members of the lab for making it a friendly and supportive environment to work in. In particular, Medini Ghodgaonkar, Stephanie Felscher, Mariela Artola, Svenja Kaden, Katja Kratz, Anja Saxer, Javier Pena Diaz and Milica Eniou for answering any questions I had and for providing exciting scientific exchanges.

A collective thank you to so many other members of the IMCR who have also provided helpful suggestions and support in any way they could. Here, Arnab Chaudhuri deserves special thanks for always being open to suggestions and questions.

Many thanks also to Odete Pereira for helping take care of the frogs in our facility which provides the eggs with which I work with. She is such a curious and observative person, I was lucky to have her around.

Last but not least I am thankful to my father for introducing me to my love of learning, I dont think I've ever seen him without a book in his hand, writing an article or assay or pondering about life.

I am also thankful for Justin Harris for always being there for me and providing me with nutritious meals throught my PhD, my brother for being so insightful about life and my family for providing me with love and support even from far away. I don't say it often enough, or probably ever, but you have been missed all these years.

I would never have known where I would end up after getting on the plane to Duesselforf more than 10 years ago and I am extremely grateful for the adding up of fateful decisions that have carried me through some of the most exciting years of my life, both personally and professionally.

10 Curriculum vitae

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RESEARCH INTERESTS

DNA repair, DNA replication, Epigenetics

EDUCATION & EMPLOYMENT

June 2009 – Present
PhD student, group Prof. Dr. Josef Jiricny, Institute of Molecular Cancer Research, University of Zurich, Switzerland.
Research interest: DNA repair, Mismatch repair
Thesis: Mismatch repair dependent processing of O6-methylguanine adducts in replicating *Xenopus* egg extracts.

August 2008 – January 2009
Practical training at the German Cancer Research Centre in the laboratory of Prof. Angel (Div. Signal-transduction)

October 2002 – May 2008
Diplom-degree in Biology at the Westfaelische Wilhelms Universitaet Muenster.
Thesis: Identification of chromatin-modification factors and genes that play a role in the non-small-cell lung cancer metastasis process using ChIP-chip

October 2001 – August 2002
Studienkolleg for foreigners in Muenster, Germany

January 2001 – June 2001
Goethe Institute for the German Language in Lima, Peru

1996-2000
High school at the Colegio Peruano Britanico (Peruvian british school) in Lima, Peru

1993-1995
Primary at the Colegio Peruano Britanico (Peruvian british school) in Lima, Peru

1990-1992
Primary at Priory Infant School in Cambridge, England

Maite Olivera Harris -- Curriculum Vitae -- 2013

SCIENTIFIC EXPERTISE

Cell biology (mammalian):
Culture and maintenance of primary and non-primary cell lines (cancerous and non-cancerous), experience in establishing cell lines stably expressing proteins of interest, expertise in cell transfection methods (including delivery of siRNAs as well as plasmids).

Biochemistry:
Profound knowledge in expression and purification of recombinant proteins (bacterial and mammalian expression systems), antibody purification, *in vitro* repair assay using heteroduplexes (for MMR), dot blot assays, EMSA, primer extension assays, and western blotting techniques. Profound knowledge of preparation and experiments done with *Xenopus* egg extracts.

Molecular biology:
General cloning techniques (including Gateway® cloning), site directed mutagenesis, quantitative RT-PCR.

Transferable skills:
Project development, organization and timely execution of projects to meet deadlines, acquisition of new knowledge (theoretical and technical know-how), scientific communications including Grant applications, presentations and scientific writing, training and supervision of team members (undergraduate students)
Language skills: Spanish (mother tongue), English (native level); German (fluent); French (Elementary, spoken)

Software/Statistical Analysis:
Microsoft Office (Word, Excel, Power Point), DNA and protein sequence analysis (Mac Vector, Fasta, ClustalW), digital image processing (Photoshop, Adobe Illustrator), and statistical data analysis: Prism GraphPad, Endnote.

GRANTS

Research Grant (2010-2011): URPP Research Project Grant, University of Zurich
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PUBLICATIONS

Maite Olivera Harris, Mariela Artola Boran, Vincenzo Costanzo, Milica Eniou and Josef Jiricny: Mismatch repair dependent processing of O6-methylguanine adducts in *Xenopus* egg extracts. (Manuscript in preparation)

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Reading fiction, historical fiction and science-fiction, movies, documentaries, competitive dance